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Clinical And Pharmacogenetic Determinants Of Plasma Factor Xa Inhibitor Systemic Exposure

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Abstract

Inhibition of blood coagulation *via* oral anticoagulant therapy is the mainstay for preventing a cardioembolic stroke in patients with atrial fibrillation (AF). Factor Xa inhibitors (FXaIs), rivaroxaban and apixaban, represent a new class of oral anticoagulants that are now widely prescribed in AF patients as an alternative to traditional warfarin therapy. An important advantage of these drugs is that routine monitoring of anticoagulation response is not necessary. Nevertheless, because of their mechanism of action, FXaI antithrombotic effect can be inferred based on the observed drug plasma concentration, with prolonged periods of elevated FXaI systemic exposure associated with increased risk for major bleeding.

Currently, there is a paucity of data relating to observed interpatient variation in FXaI plasma concentrations in the post-market clinical setting. Given that the patient population that take FXaIs in the post-market setting may vary greatly with their co-morbidities and co-medications from those within clinical trials, we hypothesized the systemic FXaI exposures achieved in these individuals may vary greatly as well, and that this interpatient variation is driven by patient-specific factors (physiology, disease-states, co-medications, genetics).

In the first study, we determined rivaroxaban and apixaban plasma concentrations in a cohort of 243 AF patients during routine clinic visits, and found their measured FXaI concentration tended to be more variable than those observed in clinical trials. Approximately 12% of patients receiving rivaroxaban and 13% of patients receiving apixaban exceeded the predicted maximum FXaI plasma concentration observed in clinical trials.

In the second study, we characterized the observed variation in FXaI concentration among our apixaban-treated AF cohort through regression analysis. Age and renal function accounted for the majority of the explained variation, while female sex and amiodarone use were also significant predictors of apixaban exposure. In addition, we demonstrated, for the first time, that 4β-hydroxycholesterol plasma concentration, as a potential biomarker for apixaban metabolism, was an independent predictor of interpatient variation in apixaban concentration.

Equally important, we noted in our second study, that a large proportion of the interpatient variation remained unaccounted for, and known common genetic variation in metabolizing enzymes and efflux transporters did not significantly predict apixaban concentration within our cohort. Therefore, we applied a custom targeted next-generation exome sequencing approach (PGxSeq) to potentially identify rare or patient-specific single nucleotide variation (SNV) in subjects with unexpectedly high drug concentration that result in aberrant function or expression of apixaban metabolizing enzymes or transporters. Following successful development and validation of PGxSeq, our exploratory analysis of twelve apixaban-treated AF subjects that were sequenced, revealed rare and common SNV(s) within the selected candidate genes.

Taken together, these studies provide insight into the factors that drive variation in FXaI plasma concentration among AF patients in routine care, and serve as a framework for future investigations regarding personalization of FXaI anticoagulant therapy.

Keywords:

Factor Xa inhibitor, apixaban, rivaroxaban, pharmacokinetics, interpatient variation, exome sequencing, single nucleotide variants, pharmacogenetics

Co-Authorship Statement

Chapter Three:

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UIS, RBK, RGT were involved in the gene selection. MG, UIS, JFR were involved in capture probe design. MG, UIS, TL, WAT, JFR were involved in acquisition, analysis, statistical analysis or interpretation of data. All authors were involved in the critical revision of the manuscript.

Dedication

To Mom, Dad, and my sister Betty

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List of Abbreviations

1000G	1000 Genomes
4β-OHC	4β-hydroxycholesterol
ABC	ATP binding cassette
AF	Atrial fibrillation
ANNOVAR	Annotate Variation
ARISTOTLE	Apixaban for reduction in stroke and other thromboembolic events in atrial fibrillation
AUC	Area-underneath-the-curve
AVERROES	Apixaban versus acetylsalicylic acid to prevent stroke in atrial fibrillation oatients who have failed or are unsuitable for vitamin K antagonist treatment
BCRP	Breast cancer resistance protein
CADD	Combined Annotation Dependent Depletion
CBR3	Carbonyl reductase 3
CES1	Carboxylesterase 1
Cmax	Maximum plasma concentration
Cmin	Minimum plasma concentration
COAG	Clarification of optimal anticoagulation through genetics trial
CPIC	Clinical Pharmacogenetics Implementation Consortium
CYP	Cytochrome P450
dbSNP137	Single Nucleotide Polymorphism database build 137
ddNTPs	Dideoxyribonucleotide-triphosphates
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide-triphosphates
DOACs	Direct-acting oral anticoagulant
DOC	Depth-of-coverage
DVT	Deep venous thrombosis
eCrCl	Estimated creatinine clearance
EMA	European Medicines Agency

ENGAGE AF-TIMI 48	Effective Anticoagulation with Factor Xa Next Generation in Atrial Fibrillation–Thrombolysis in Myocardial Infarction 48
EU-PACT	European pharmacogenetics of anticoagulant therapy trials
ExAC	Exome Aggregation Consortium
FDA	US federal drug administration
FIX	Factor IX
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXaI	Factor Xa inhibitors
FXaIs	Factor Xa inhibitors
FXI	Factor XI
FXR	Farnesoid X receptor
GIFT	Genetic Informatics Trial
GST	Glutathione S-transferase
GWA	Genome-wide association
INR	International normalized ratio
LAA	Left atrial appendage
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDLR	Low-density lipoprotein receptor
MAF	Minor allele frequency
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
NR	Nuclear receptor
OAC	Oral anticoagulants
P-gp	P-glycoprotein
PAR	Protease-activated receptor
PCR	Polymerase chain reaction
POR	P450 oxidoreductase

PT	Prothrombin time
QCs	Quality controls
RCT	Randomized control trials
ROCKET AF	Rivaroxaban once daily oral direct factor Xa inhibition compared with vitamin K antagonism for prevention of stroke and embolism trial in atrial fibrillation
SIFT	Sorting Intolerant from Tolerant
SLC	Solute carrier
SNPs	Single nucleotide polymorphisms
SNV	Single nucleotide variation
SSE	Stroke and systemic embolism
TDM	therapeutic drug monitoring
TF	Tissue factor
TTR	Time in therapeutic range
UCSC	University of California Santa Cruz
UGT	UDP glucuronosyltransferases
UHPLC-MS/MS	Ultra-high-pressure liquid chromatography-tandem mass spectrometry
UTR	Untranslated regions
VKORC1	Vitamin K epoxide reductase enzyme
VTE	Venous thromboembolisms
vWF	Von willebrand factor
WHO	World health organization

1 Introduction

1.1 Blood coagulation

Blood coagulation as part of a process known as hemostasis, is the body's physiological response to blood vessel injury. Upon damage to blood vessels, a chain of pro-inflammatory and wound-healing processes begin. This includes the formation of blood clots that are rich in platelets, red blood cells, as well as fibrin protein, which prevents the loss of blood and allow recovery to commence. To date, there are two prominent models that conceptualize coagulation; the "cascade" model was proposed first in the 1960s (1, 2), while the "cell-based" model is more recent (3).

1.1.1 Cascade model

The cascade model depicts coagulation as a process that is directed and controlled by a series of interactions between protease zymogens, enzymes, and cofactors known as coagulation factors that lead to the generation of thrombin and fibrin (**Figure 1-1**). Although this model accurately depicts the interactions between individual coagulation factors, the concept that coagulation is segregated into two distinct pathways, the intrinsic and extrinsic pathways, provides an inadequate explanation of hemostasis as it occurs *in vivo*. This statement is supported by the observation that the factor VII protease/tissue factor complex of the extrinsic pathway is capable of activating factor IX of the intrinsic pathway (4). Additionally, the downstream protease, thrombin has been shown to activate factor XI of the intrinsic pathway (5). These findings were among the key steps that led to the understanding that the "intrinsic" and "extrinsic"

pathways are more interdependent than previously described.

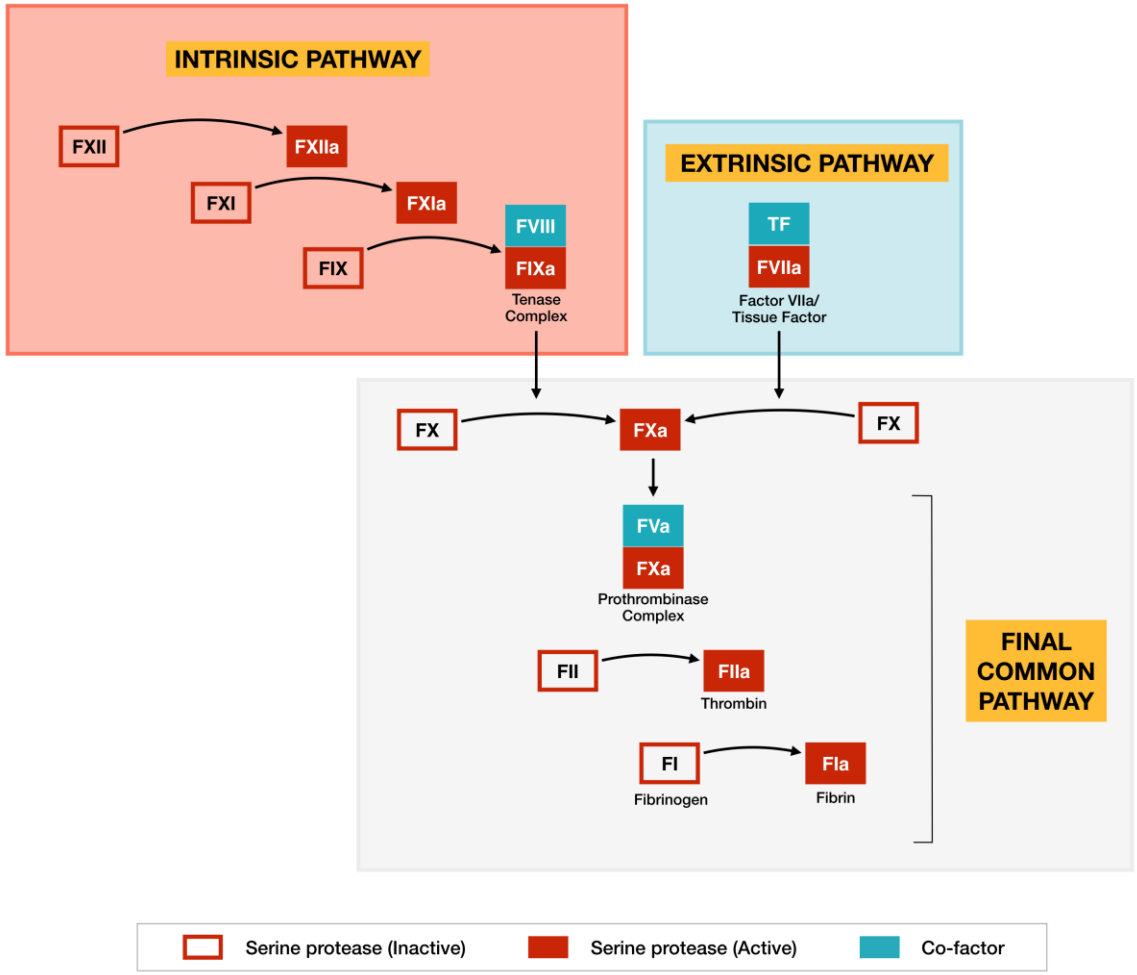

Figure 1-1. Cascade model of coagulation.

Abbreviations: TF , tissue factor

1.1.2 Cell-based model

The cell-based model of blood coagulation is viewed as occurring in three successive non-exclusive phases: initiation, amplification, and propagation (3)

Figure 1-2).

The initiation phase begins when tissue factor (TF, a membrane-bound protein) from extravascular cells become exposed to blood constituents at the injury site. Circulating factor VII is activated upon exposure to extravascular TF and then forms a complex with TF (6), which subsequently activates factors X and IX. As circulating platelets are exposed to the site of injury they adhere to collagen found in the extravascular matrix and become partially activated. A consequence of platelet activation is the granular secretion of partially activated factor V (7), which is a cofactor of the activated factor X (FXa) protease. FXa can also further activate factor V (7). Activated factor V (FVa) can combine with FXa on the cell surface of TF-expressing cells in the presence of calcium to produce a small burst of thrombin protease at the injury site (8).

The thrombin generated during initiation amplifies the procoagulant state, by activating platelets and coagulation factors, thereby setting the stage for the assembly of procoagulant complexes on the surface of platelets to commence large-scale thrombin generation. Thrombin is a potent activator of platelets via the protease-activated receptors (PAR), where the enzyme cleaves a specific N-terminus peptide sequence from PARs to unmask a tethered ligand that stimulates the receptor to induce downstream procoagulant responses. The initial burst of thrombin also activates coagulation factors that are known to be part of the "intrinsic" pathway, particularly factor XI (5, 8) and factor VIII (9). These activated factors along with the aforementioned FVa are then recruited to the surface of platelets, ready for the propagation phase.

During propagation, the initial burst of activated factor IX (FIXa) and factor X

(FXa) proteases combine with their respective co-factors on the surface of platelets to assemble tenase (FIXa/FVIIIa) and prothrombinase (factor FXa/FVa) complexes. The overall shift in procoagulant activity from TF-expressing cells towards platelets is facilitated by an increased exposure of negatively charged phospholipids (e.g., phosphatidylserine) on the outer membrane leaflet of platelets that is secondary to their activation (10). The presence of anionic phospholipids along with calcium is necessary for the assembly and function of coagulation factors (11). Furthermore, given that each activated protease is required for the subsequent activation of a downstream protease (e.g., FXIa activates FIX, FIXa is incorporated into a tenase (FIXa/FVIIIa) complex which activates FX etc.) the close proximity of these enzymes on the platelet surface facilitates the rapid burst of thrombin production required for a hemostatic fibrin clot.

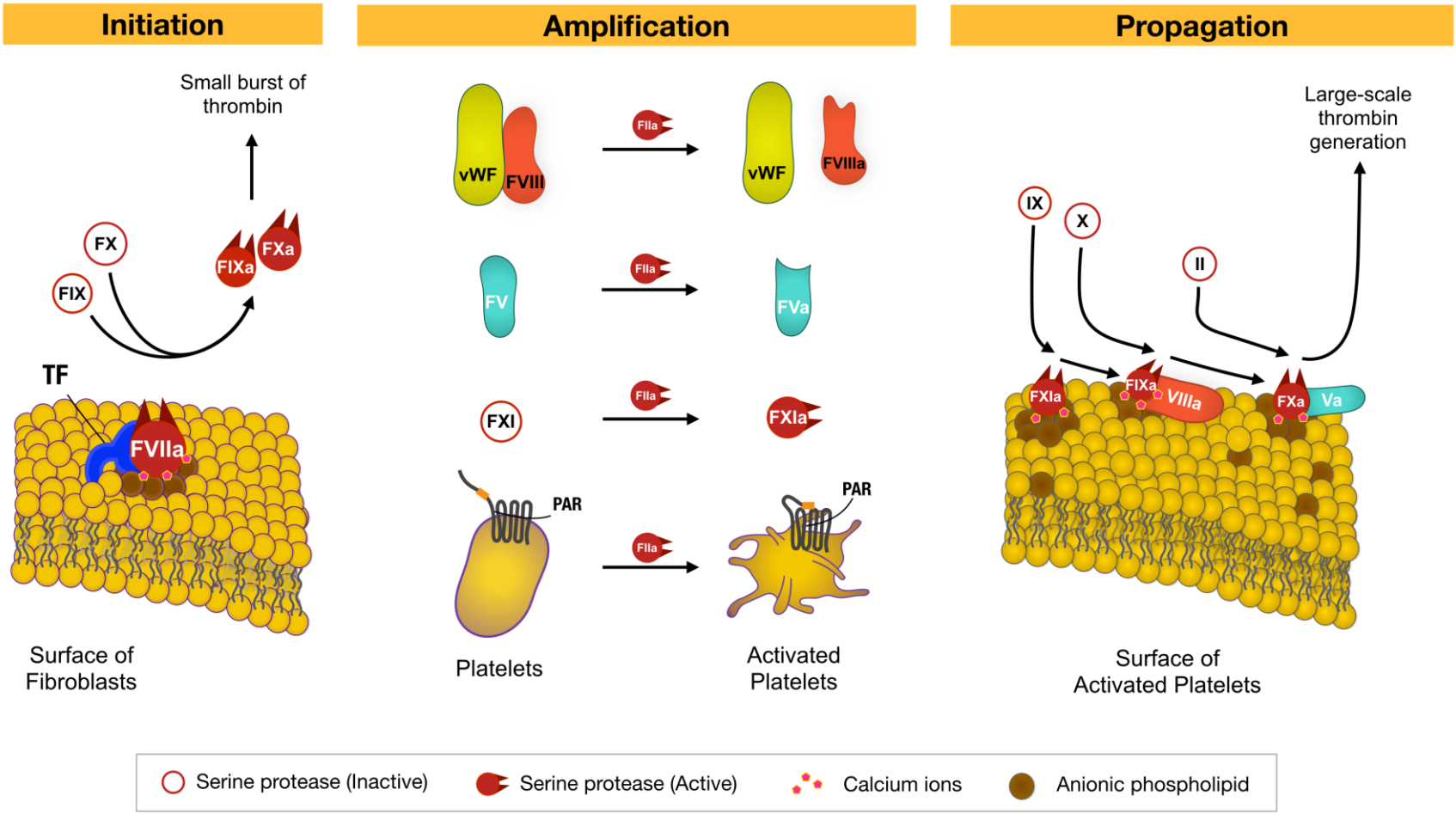
Initiation
Amplification
Propagation
Small burst of thrombin
FX
FIX
FIXa
FXa
TF
FVIIa
Surface of Fibroblasts
vWF
FVIII
FIIa
vWF
FVIIIa
FV
FIIa
FVa
FXI
FIIa
FXIa
PAR
FIIa
PAR
Platelets
Activated Platelets
Large-scale thrombin generation
IX
X
II
FXIa
FIXa
VIIIa
FXa
Va
Surface of Activated Platelets
Serine protease (Inactive)
Serine protease (Active)
Calcium ions
Anionic phospholipid

Figure 1-2. Cell-based model of coagulation.

In the cell-based model, coagulation occurs in three subsequent non-exclusive phases: initiation, amplification, propagation. Trace amounts of thrombin are formed during the initiation phase. In the amplification phase, the thrombin produced potentiates the procoagulant state through cleavage activation of coagulation factors (FV, FVIII, FXI), and PAR which is located on the surface of platelets. During the propagation phase, the activated proteases and their co-factors form an assembly of coagulation complexes on the surface of activated platelets, where their close proximity to one another facilitates the large-scale thrombin generation required for a hemostatic clot. Abbreviations: PAR, protease-activated receptors; TF, tissue factor; vWF, von Willebrand

1.2 Thrombosis

Maintaining normal blood fluidity requires a complex balancing of the procoagulant (thrombus producing) and anticoagulant (thrombus inhibiting) processes. An imbalance of these opposing processes gives rise to the potential for the formation of pathological thrombi within the blood vessel. Thrombi can cause local obstruction of blood flow or branch off and travel downstream to embolize elsewhere in the circulatory system in a process known as thromboembolism. Thromboembolisms that obstruct blood flow to vital organs such as the heart, brain, or lung can cause life-threatening complications. The triggering events that lead to occlusive thrombus formation are consequence of one or more of following: abnormal changes to the vascular wall, circulatory stasis, or a hypercoagulable state (**Figure 1-3**). This combination of events was first elucidated by Rudolf Virchow in the 1700's and is known as Virchow's "triad" (12).

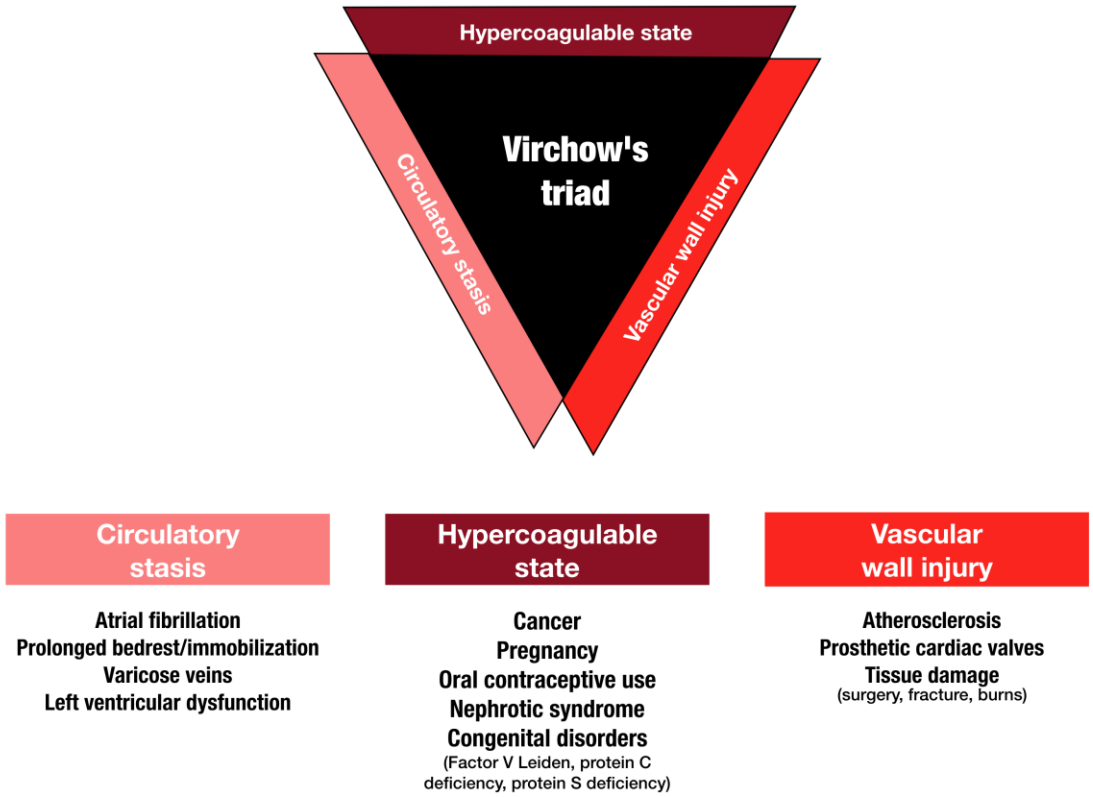

Figure 1-3. Virchow's triad.

1.2.1 Venous thrombosis

The vascular environment plays an important role in the type of thrombus formed, as well as in determining the associated clinical complications. Venous thrombosis most commonly occurs within the deep veins of the leg where it is known as deep venous thrombosis (DVT). It can be caused by stasis in venous circulation (e.g. prolonged bedrest), as well as impaired local regulation of anticoagulant processes (e.g. protein C and S deficiency). Clinical complications of DVT include edema and congestion in the vascular beds distal to an obstruction; however, DVT is most problematic for its capacity to originate pulmonary embolisms that cause death.

1.2.2 Arterial thrombosis

Typically, arterial thrombosis occurs following the rupture of an atherosclerotic plaque in the presence of fast moving blood, with thrombus formation highly dependent on platelets. A notable exception to this occurs in the case of atrial fibrillation (AF), since AF-related thrombi are formed under low blood flow and are dependent upon the activation of the coagulation cascade. The major complications of arterial thrombosis include the obstruction of coronary and cerebral arteries causing cardiac ischemia (angina, myocardial infarction) and ischemic stroke, respectively.

1.3 Thrombogenesis in the setting of atrial fibrillation

Atrial fibrillation is the most common pathological cardiac arrhythmia and is most prevalent among the elderly (13). In 2010, the estimated number of men and women affected by AF worldwide were 20.9 and 12.6 million, respectively (14). Cardiogenic cerebral thromboembolisms resulting in ischemic stroke have long been strongly associated with AF, dating back to the Framingham study in the early 1990s, which reported a 5-fold greater incidence of stroke in those with AF as compared with disease-free subjects (13).

Cardiogenic emboli in the setting of AF are also thought to be triggered by the fulfillment of Virchow's triad (15). During episodes of AF, the atrial myocardial tissue experiences uncoordinated activation leading to ineffective contractions and reduced cardiac flow, which promotes blood stasis, particularly in the left atrium and left atrial appendage (LAA). The LAA, a long narrow inlet within the left atrium, is believed to be a major source of cardiogenic emboli leading to ischemic stroke (16, 17). In addition to stasis, structural changes in the form of atrial dilation and endocardial dysfunction are also attributed to AF (16, 18-20). Finally, there is evidence of abnormal changes in blood constituents that are indicative of a hypercoagulable state (15), thereby fulfilling Virchow's triad. For example, patients with AF exhibit elevated levels of the fibrin degradation product, D-dimer (21, 22), and von-Willebrand factor (23, 24) compared to patients without AF. Inflammatory cytokines (e.g. high-sensitivity C-reactive protein (25) and interleukin 6 (26)) as well as growth factors (e.g. vascular endothelial growth factor (23, 27)) that stimulate TF production (28, 29), have been also found to be elevated in patients

with AF, suggesting that inflammation as well as enhanced growth factors could also be contributing to the hypercoaguable state in AF patients.

1.4 Stroke prevention in patients with atrial fibrillation

Oral anticoagulants (OACs) are the recommended preventative therapy for reducing stroke risk in patients with AF (30-32). As a class, anticoagulants function by hindering fibrin formation either through indirect or direct inhibition of coagulation factor(s). Compared to antiplatelets, which inhibit platelet activation or aggregation, OACs are more effective at reducing stroke and mortality incidence in AF patients (33, 34). The effectiveness of OACs over antiplatelets can be attributed to the fibrin-rich, low-platelet nature of AF-related thrombi (35).

1.4.1 Therapeutic interventions

The recommendation for use of OACs in the setting of AF is based on a patient's assessed stroke risk (31), which is increased by the presence of additional clinical factors such as advanced age, hypertension, and diabetes mellitus (36). Therefore, prediction formulas such as $CHADS_2$ (36) and CHA_2DS_2-VASc (37) (defined in **Table 1-1** and **Table 1-2**) have been developed to stratify patients with AF in order to identify those that might benefit from receiving anticoagulant therapy. For example, according to the 2016 Canadian Cardiovascular Society guidelines for management of AF patients, OAC therapy is recommended for AF patients that are ≥ 65 years of age or for younger patients with $CHADS_2$ score ≥ 1(38).

Table 1-1. $CHADS_2$ stroke risk stratification schema for patients with atrial fibrillation

$CHADS_2$		Points†
C	Congestive heart failure	1
H	Hypertension	1
A	Age ≥75	1
D	Diabetes mellitus	1
S	Prior stroke or transient ischemic attack	2

† Based on the number of risk factors an individual possesses these points are summed to calculate their $CHADS_2$ score value.

Table 1-2. CHA_2DS_2-VASc stroke risk stratification schema for patients with atrial fibrillation.

$CHA_2DS_2VAS_c$		Points†
C	Congestive heart failure (Left ventricular ejection fraction ≤40%)	1
H	Hypertension	1
A	Age ≥75 years	2
D	Diabetes mellitus	1
S	Prior stroke/transient ischemic attack/thromboembolism	2
V	Vascular disease	1
A	Age 64-74 years	1
S	Female sex	1

†Based on the number of risk factors an individual possesses these points are summed to calculate their $CHA_2DS_2VAS_c$ score value.

1.4.2 Warfarin

Warfarin was the first OAC widely used as a prophylactic for stroke prevention in patients with AF. Its use is associated with a 64% reduction in stroke incidence compared to placebo (39). Warfarin indirectly depletes the biologically active supply of key vitamin K-dependent clotting factors (II, VII, IX, X, protein C and S) through its action as a Vitamin K epoxide reductase antagonist. While warfarin is highly effective at reducing stroke risk, major bleeding remains a significant safety concern with its long-term use.

1.4.3 Direct-acting oral anticoagulants

1.4.3.1 Phase III clinical trial data

The advent of the next generation of OACs was enabled by the discovery of the crystal structures of the key coagulation factors thrombin (40) and factor Xa (41). Small molecule inhibitors called direct-acting oral anticoagulants (DOACs) were developed to directly target these rate limiting enzymes. Four pivotal randomized control trials (RCT) that compared the efficacy and safety outcomes of each of these drugs to warfarin were published between 2009 and 2013 (42-45). Dabigatran, the first thrombin inhibitor to be approved, was found to be equally efficacious as warfarin for prevention of stroke and systemic embolism (SSE) in AF patients and had comparable adverse bleeding rates. This was followed by the approval of the factor Xa inhibitors rivaroxaban, apixaban, and edoxaban. Compared to warfarin, apixaban was superior in reducing SSE events and had lower rates of bleeding, while rivaroxaban and edoxaban had comparable rates of SSE. The rates of bleeding were lower for edoxaban when compared to warfarin, however were comparable between rivaroxaban and warfarin. Further meta-analysis of pooled data from

all four trials found that the greatest benefit of the DOACs class was a substantially reduced rate of intracranial hemorrhage, which is a potentially fatal complication of OAC treatment, while the greatest detriment was a higher rate of gastrointestinal bleeding (46). Thus overall, RCT data concluded that DOACs offer a favourable balance of safety and efficacy compared to warfarin.

1.4.3.2 Dosing and administration

The initial dose selection is straightforward for DOACs. Patients are given either the standard dose or a reduced dose based upon meeting specific clinical criteria such as renal impairment, low body weight, and interacting medications. The recommended dose of dabigatran is 150 mg twice daily or 110 mg twice daily if patients are aged ≥ 80 years (47). Rivaroxaban is to be taken with food at a standard dose of 20 mg once daily, unless patients have moderate renal impairment with an estimated creatinine clearance ranging between 30 and 49 mL/min (48). The recommended dosage for apixaban is 5 mg twice daily, or 2.5-mg twice daily for patients fulfilling two of the three following clinical criteria: age ≥ 80 years, serum creatinine value ≥ 133 mmol/L, and weight ≥ 60 kg (49). Edoxaban has a recommended dose of 60 mg once daily or 30 mg once daily in patients with one or more of the following clinical criteria: moderate renal impairment as defined above, body weight ≤ 60 kg, or a taking an interacting medication such as a P-glycoprotein inhibitor (50).

1.5 Clinical and genetic determinants of optimal oral anticoagulant therapy

Given the mechanisms by which OACs elicit their effects, differences in drug response are a concern for adverse reactions. The efficacy of OACs in reducing stroke or systemic embolic events is counter-weighed by the risk of major bleeding events, including bleeds that result in death by exsanguination, symptomatic bleeding into critical sites or organs (e.g. intracranial or gastrointestinal), a hemoglobin drop ≥2 g/dL, or the necessity of a blood transfusion. Therefore, achieving balanced anticoagulation is critical for maximizing the safety and efficacy of OACs.

1.5.1 Warfarin

The earliest attempts at long-term warfarin use were complicated by unacceptably high bleeding rates (51, 52). Subsequently, it was found that lower intensity anticoagulation regimens monitored carefully by hemostatic testing using the prothrombin time (PT) test could be as effective (**Figure 1-4**), but safer than high-intensity regimens (53, 54), improving the safety of warfarin use in patients. PT test measures the clotting tendency of blood determined as the time required for a blood sample to clot, specifically evaluating the extrinsic pathway of coagulation (factors I, II, V, VII, X), where warfarin treated samples would have longer PT times, then untreated samples. In the 1980s, prothrombin international normalized ratio (INR) was introduced by the World Health Organization (WHO), which globally standardized the PT test for assessing anticoagulation. Today, warfarin dosing is based on achieving a target INR value of between 2.0 and 3.0 for AF patients (54). However, in order to achieve this therapeutic range in patients, prescribers

must accommodate an up to 20-fold variation in warfarin dose that exists between individuals. Aside from demographic (age, sex, weight) and clinical variables (renal or hepatic disease, dietary, interacting medication), there is a significant contribution from genetic variation within the proteins involved in warfarin's metabolic and/or drug response pathway that complicate achieving a therapeutic warfarin dose.

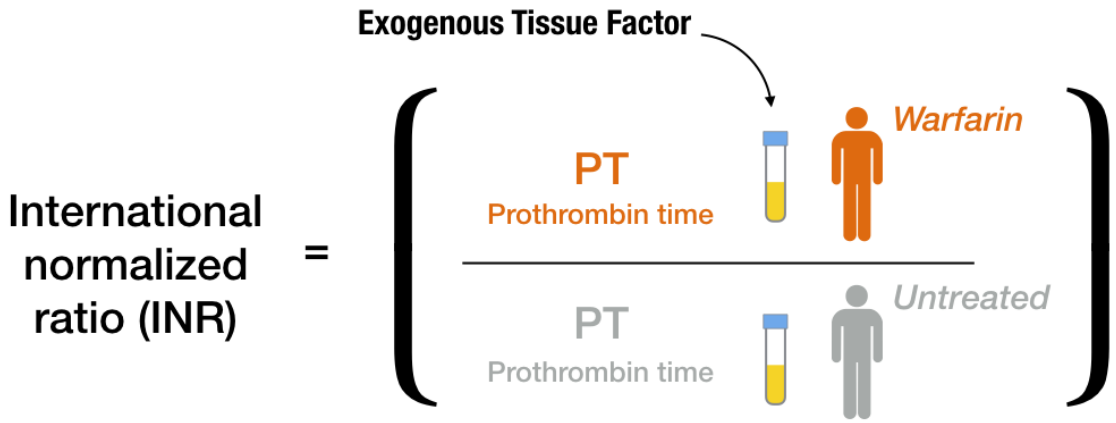

Figure 1-4. International normalized ratio and prothrombin time

International normalized ratio (INR) is expressed as the relationship between the prothrombin time of a warfarin-treated blood sample over the prothrombin of an untreated sample. INR values between 2.0 and 3.0 provide greatest therapeutic benefit. INR values less than 2 and greater than 4 are associated with increased risk of stroke and hemorrhage, respectively.

1.5.1.1 Mechanistic determinants of warfarin dose response

Warfarin is administered as a racemic mixture of two active enantiomers (*R*- and *S*-isomers) that are rapidly absorbed from the gastrointestinal tract at high oral bioavailability (product monograph). After absorption, the drug accumulates in the liver and functions as an inhibitor of the Vitamin K epoxide reductase enzyme (VKORC1) with the *S* isomer as the stronger antagonist (55, 56). Warfarin is almost entirely excreted through the urine in the metabolite form (57). The two enantiomers undergo metabolism by different cytochrome P450 (CYP) enzymes (58), with the inhibition of *S*-warfarin metabolism being of greater clinical relevance.

The more potent enantiomer, *S*-warfarin, is metabolized by CYP2C9 into its inactive form *S*-7-hydroxywarfarin (58). However, the *CYP2C9* gene has several genetic variations, of which the *CYP2C9*2* and *CYP2C9*3* polymorphisms show decreased enzymatic hydroxylation of *S*-warfarin *in vitro* (59, 60). The allele frequencies for *CYP2C9*2* (c.430 C>T) and *CYP2C9**3 (c.1075 A>C) are approximately 12.5% and 8.5% in Caucasian populations (61), although these numbers vary based on ancestry. Genotyping for these alleles have demonstrated that variant carriers have reduced *S*-warfarin clearance (57), reduced warfarin dose requirements (62, 63), and increased risk of over anticoagulation. In a meta-analysis of nine studies including 2275 patients, variant carriers of *CYP2C9**2 and *CYP2C9**3 had an average dose reduction of 17% and 37%, respectively, compared to *CYP2C9**1 (wildtype) (64).

Warfarin elicits its anticoagulant effect on the enzyme VKORC1, which is involved in the cyclic interconversion of Vitamin K (**Figure 1-5**) as part of the physiological recycling of Vitamin K_1 dihydroquinone (65). Vitamin K_1 dihydroquinone (VKH2) is an

essential cofactor in the post-translation carboxylation of N-terminal glutamate residues on Vitamin-K dependent coagulation proteins (65). These proteins, which include coagulation factors II, VII, IX, and X as well as anticoagulant factors protein C and protein S, require carboxylation to render them biologically active (65). Gamma-glutamyl carboxylase is responsible for carrying out this reaction using VKH2, CO_2, and O_2. VKH2 is converted to Vitamin K epoxide as a product of the reaction. VKORC1 is then responsible for the chemical reduction of vitamin K epoxide to vitamin $K_{1,}$ an intermediate that is further reduced to vitamin K_1 dihydroquinone (65). Therefore, by inhibiting VKORC1, warfarin impedes the vitamin K cycle and depletes the biologically active supply of vitamin K dependent coagulation factors.

Identification of the *VKORC1* gene (66) led to investigations of the effect of *VKORC1* genetic variation on warfarin dosing. Several studies identified a single nucleotide polymorphism (c.-1639 G>A, *2) located within the promoter region of the gene that was associated with dose response to warfarin as measured by INR (67-70). Carriers of this polymorphism were more sensitive to warfarin and required a lower dosage to achieve the target INR (67-70), independent of the *CYP2C9* genetic variation. The molecular mechanism behind this warfarin sensitivity is believed to be at the transcriptional level, as demonstrated *in vitro* with the -1639A allele having reduced *VKORC1* promoter activity (68, 69).

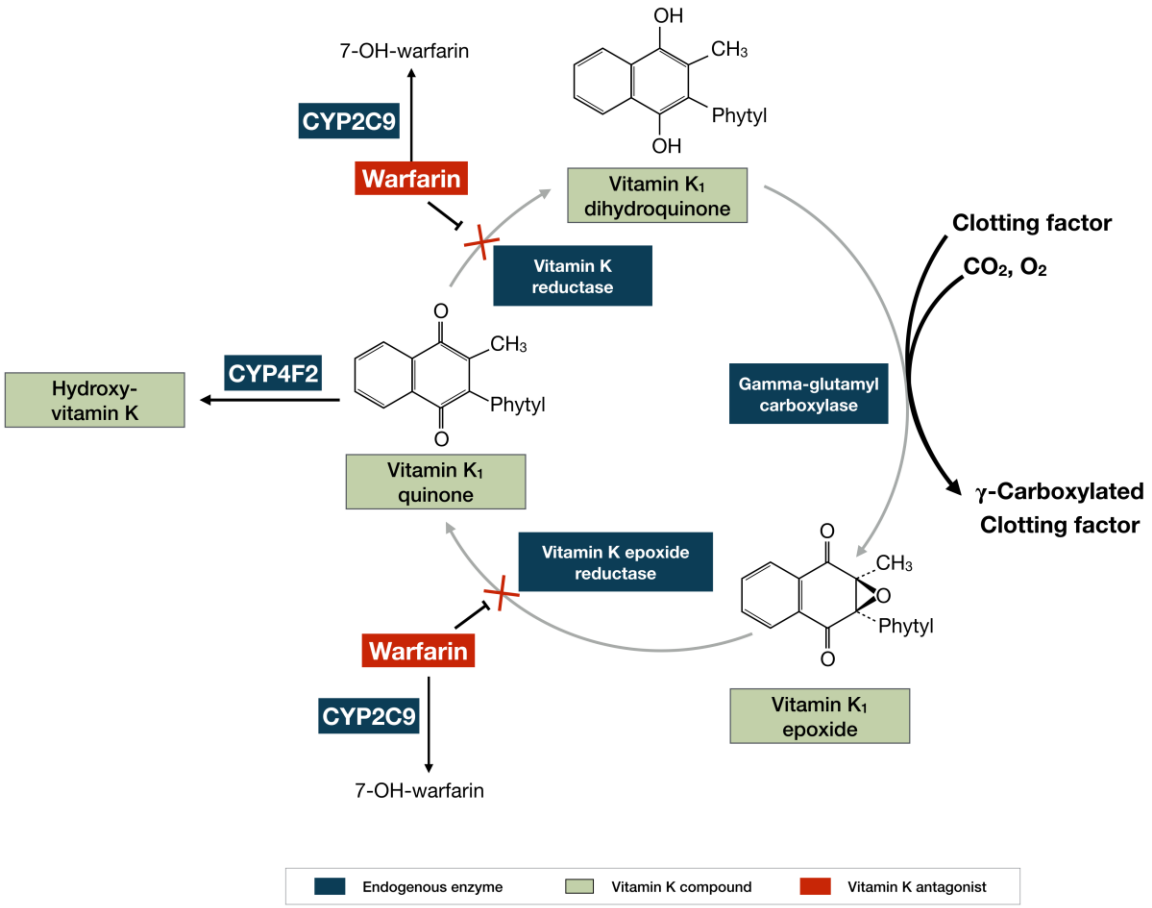

Figure 1-5. Vitamin K cycle, and the involvement of warfarin, Vitamin K epoxide reductase, CYP2C9, and CYP4F2.

Warfarin effectively lowers the supply of biologically active coagulation factors by inhibiting the conversion of Vitamin K epoxide (oxidized form) to Vitmain K dihydroquinone (reduced form). Vitamin K dihydroquinone is a cofactor required for the post-translation carboxylation of amino-terminus residues on key coagulation factors (II, VII, IX, X); a process that is essential for rendering these proteins biologically active. Vitamin K epoxide (oxidized form) is product of this carboxylation reaction, and Vitamin K epoxide reductase is responsible for converting the oxidized product back into Vitamin K dihydroquinone. CYP4F2 is a vitamin K_1 oxidase, and is believed to be involvement in the removal of Vitamin K_1 quinone from the Vitamin K cycle. Abbreviations: CYP, cytochrome P450 enzyme.

Studies using multiple regression analysis to examine the combined effect of *CYP2C9* and *VKORC1* polymorphisms, as well as clinical variables (age, sex, body surface area, interacting medications), found that the proportion of inter-individual variability in warfarin dosage accounted for by genetic factors was greater than that explained by clinical variables (36, 71). In a study designed to develop a warfarin-dosing algorithm, authors were able to explain 53-54% of the variability in warfarin doses using genetic factors along with clinical variables (age, body surface area, interacting medications, and indication for warfarin use), while clinical variables alone explained only 17-22% of dose variability (72). This observation was replicated in a later study where *CYP2C9**2 and *3 polymorphisms explained a combined 12% of the variation in warfarin dose, while the *VKORC1**2 polymorphism explained 30%, and an additional 15% was explained by clinical variables (71).

Unbiased detection of statistical associations between specific regions in the genome and a clinical outcome of interest can be accomplished using genome-wide association (GWA) studies. In two GWA studies that screened up to 550,000 SNPs spanning the whole genome in sample sizes of 300 and 1000 Caucasians, it was found that the statistical signals emanated from the *VKORC1* gene, followed by *CYP2C9* gene (73, 74).

1.5.1.2 Randomized control trial evidence for genomic-guided warfarin therapy

The large amount of evidence on the impact of genetic factors on warfarin dosing has given rise to a number of pharmacogenomic-guided dosing algorithms that incorporate *CYP2C9* and *VKORC1* genotypes along with clinical variables for predicting warfarin initiation and maintenance doses (72, 75, 76). However, determining the net clinical benefit of the implementation of such algorithms into clinical practice has been challenging with RCT bearing positive and neutral results for genomics-guided dosing.

The Clarification of Optimal Anticoagulation through Genetics (COAG) trial and two European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) trials were published in 2013. These trials measured the benefit of genetic-guided warfarin dosing in patients with AF or venous thromboembolism using time in therapeutic range (TTR) as the primary outcome (77-79). TTR is a useful proxy measure for clinical risk of bleeding (over-anticoagulation) or thrombosis (under-anticoagulation) as these studies were not sufficiently powered to detect differences in these outcomes directly. Two of the trials compared genomics-guided dosing with a clinical variables algorithm and found no statistical difference in the percentage TTR at the end of their respective follow up periods of four or twelve weeks (77, 78). The third trial compared a genomics-guided algorithm to standard dosing (no algorithm), which is more representative of usual care, and found significant benefits to using the genomics-guided algorithm (79). The authors reported a greater TTR, fewer incidences of elevated INR, and shorter times to therapeutic range when using their pharmacogenetic algorithm (79).

In 2017, the Genetic Informatics Trial (GIFT) compared genotype-guided dosing *versus* a clinical algorithm in initiating warfarin therapy for prevention of venous thromboembolisms (VTE) in older subjects (≥65 years) following knee or hip surgery (80). The primary outcome was a composite of INR ≥4, major bleeding, VTE, or death. Genomics-guided dosing resulted in a statistically significant reduction of the composite primary outcome. Notably, the authors of this study included an additional variant, *CYP4F2**2, in their genotyping testing.

The conflicting results from these RCTs have instigated criticism regarding the design and execution of these studies as well as debate surrounding the value of genomics-guided warfarin dosing (81). For instance, the 2013 EU-PACT trial had positive findings using standard dosing (with dose tailored only to age) as their control arm rather than a clinical algorithm, essentially testing a hypothesis that differs from the other trials. In addition, it is well known that there are ethnic differences in the prevalence of *CYP2C9* and *VKORC1* variant genotypes (82, 83), underscoring the importance of tailoring the variants included within a pharmacogenomics-guided dosing algorithm to account for prevalence of those alleles within the tested ethnic population. In the negative COAG trial, nearly 30% of participants were of African descent, yet the variants tested did not include *VKORC1* and *CYP2C9* variants that are more prevalent and predictive of warfarin dosing among African populations. Some authors believe this may have negatively impacted the genomic-guided arm by overdosing some African patients (81). Lastly, both the positive EU-PACT trial and the GIFT trial performed preemptive genotyping, where the variant status was known prior to initiating warfarin. Conversely, in the COAG trial only 45% of patients within the genomics-guided group had their variant status available for

incorporating into the dosing algorithm prior to receiving their first dose. Some authors argue that the greatest benefit for genomics-guided dosing is achieved during the initiation phase of warfarin dosing (81). Overall, it appears that the greatest clinical benefit is achieved when using genomic-guided algorithms for the initial warfarin dose selection in patients preemptively genotyped for variants tailored to their ancestry (84).

1.5.2 Next generation of oral anticoagulants

Not surprisingly, given the issues surrounding warfarin, direct-acting oral anticoagulants (DOAC) were developed and are now approved as an alternative therapy for stroke prevention in patients with AF, as well as prevention of venous thromboembolisms. Since their approval, the use of these drug in AF patients has rapidly increased (85, 86), owing to their ease of prescribing and management. While warfarin prescribing was complicated by routine INR testing and a high interpatient variability in the therapeutic dosage, DOACs offer fixed dosing (up to 2-fold) without the need for routine monitoring. Although DOACs appear to be safer than warfarin, major bleeding is still a complication associated with DOAC use. Furthermore, the patient population that take DOACs in the post-market setting may vary greatly with their co-morbidities and extensive co-medications from the often idealistic settings that exists within clinical trials. As prescribing trends in clinical practice continue to favour the use of DOACs, the number of bleeding events associated with them is likely to increase as well. Identifying factors that increase the DOAC-associated bleeding risk in patients before prescribing these drugs can help to mitigate adverse drug events. Factors that increase DOAC bleeding risk can be categorized into the following: advanced age (≥75 years), co-medication with pharmacodynamic interactions, medical conditions or

procedures with pre-existing bleeding risks, as well as factors that increase DOAC plasma concentrations.

Although there are a number of DOACs currently available on the market, the remainder of this thesis will be focused on the factor Xa inhibitors, rivaroxaban and apixaban, as the patient population we investigated were primarily prescribed these DOACs.

1.5.3 Factor Xa Inhibitors

1.5.3.1 Significance of factor Xa inhibitor drug exposure

The factor Xa inhibitors (FXaIs), rivaroxaban and apixaban, have a pharmacological effect that is closely correlated to their blood concentration (87-90). Taken orally, FXaIs are absorbed at the level of the intestine, then enter the systemic blood circulation (at 50-100% bioavailability see Table 1-3) where they act on their circulating target, which is the activated coagulation protein factor Xa (FXa). Through reversibly binding to the active site of FXa with high affinity, these drugs effectively inhibit the catalytic activity of both free and prothrombinase-bound FXa (91, 92). From a pharmacological standpoint, this ability of rivaroxaban and apixaban to *directly* inhibit factor Xa, allows them to have a rapid onset and offset of action that is measured in hours (87, 89), unlike warfarin, which takes days to produce therapeutic efficacy (57). Inhibition of factor Xa reduces thrombin generation and consequently hinders clot formation given thrombin's effects on fibrin formation and platelet activation. *In vitro* studies investigating factor Xa activity at varying concentrations of FXaI in human blood plasma have shown a positive relationship between increasing rivaroxaban and apixaban concentration and factor Xa inhibition (89, 93). Animal models

used to study FXaI antithrombotic efficacy have shown that a reduction in thrombus formation correlates with increased FXaI dose (92, 93). Hemostatic testing of FXaI-treated patient plasma samples to measure the sample's ability to clot under standard conditions also show a concentration-dependent relationship, with longer clotting times as drug concentrations increase in plasma (89, 93).

The potential relationship between the circulating concentration of FXaI and harm such as major bleeding in contrast to its efficacy in stroke prevention in AF or VTE in total knee/hip replacement was noted in the Food and Drug Administration (FDA)'s clinical pharmacology and biopharmaceutics reviews documents, which reported that elevated rivaroxaban and apixaban exposure were associated with increased risk of bleeding (94, 95). Similarly, the more recently approved FXaI, edoxaban, was also found to have this positive relationship between bleeding events and plasma concentration (96). Consequently, patient factors that increase FXaI plasma concentration is among the listed factors to influence risk of hemorrhage (48, 49).

Table 1-3. Summary of the pharmacokinetic parameters for rivaroxaban and apixaban in healthy subjects.

Parameter	Rivaroxaban	Apixaban
Mechanism of action	Factor Xa inhibitor	Factor Xa inhibitor
Bioavailability, %	>80* (97)	50 (98)
T_{max}, hours	2-4 (48)	1.5-3.3 (88)
$T_{1/2,}$ hours	11-13 (48)	12 (49)
Protein binding, %	92-95 (48)	87-93 (99)
Total Clearance, L/h †	10 (48)	3.3 (49)
Renal clearance, % (of total clearance) †	30-40 (48)	27 (98)
V_{ss} , L	50 (48)	21(98)
Food effect	Increase AUC by 39% (97)	None (88)
Renal excretion, % ‡	36 (100)	22 (101, 102)
Fecal excretion, % ‡	7 (100)	34 (101, 102)
CYP-mediated metabolism	CYP3A4/5, CYP2J2 (103)	CYP3A4/5 (104)
Drug transporters	P-gp, BCRP (105)	P-gp, BCRP (106)

Abbreviation: AUC, area-under-the-curve; BCRP, breast cancer resistance protein; C_{max}, maximum concentration; FXa K_i, inhibition constant of Factor Xa enzyme; $T_{1/2}$, terminal elimination half-life; T_{max}, time at maximum concentration; P-gp, P-glycoprotein; V_{ss} , volume of distribution at steady-state. (†) After intravenous administration; (‡) Percent recovery of unchanged radio-labelled drug; (*) Bioavailability is 80-100% when taken with food, or 66% without food.

1.5.3.2 Determinants of FXaI exposure

Phase I pharmacokinetic studies have explored the impact of renal function, advanced age, and extremes of weight on rivaroxaban and apixaban exposure, often measured as the area underneath the plasma concentration time profile or area-underneath-the-curve (AUC). Kidney function is a strong determinant of FXaI drug exposure as both drugs undergo urinary excretion as part of their elimination process. Individuals with impaired renal function exhibit reduced total clearance and increased drug exposure, such that subjects with mild, moderate, and severe renal impairment were associated with ~16%, ~29%, and ~44% higher AUC values for apixaban and ~44%, ~52%, and ~64% for rivaroxaban, respectively (107, 108). Advanced age has been shown to influence FXaI total clearance resulting in modest increases in drug exposure in subjects ≥65 years for apixaban (32% higher AUC compared to those aged 18-40 years) (109) and subjects >75 years for rivaroxaban (41% higher AUC compared to those aged 18-45 years) (110). However, differences in renal function between these age groups is likely to contribute to these effects. Body weight greater than or equal to 120 kg has been shown to lower the apixaban AUC by 23%, while body weights less than or equal to 50 kg have a 20% increase in apixaban AUC (111). In comparison, extremes of weight appear to have minimal effect on rivaroxaban exposure (112).

Modulation of the metabolizing enzymes or transporters that are responsible for apixaban and rivaroxaban disposition can alter the drug concentrations achieved in the blood. FXaI drug metabolism is mediated primarily through the oxidative cytochrome P450 enzymes CYP3A4/5 and CYP2J2, although the latter applies only to rivaroxaban (103, 104). *In vitro* studies suggest rivaroxaban and apixaban are substrates to the efflux

transporters, P-glycoprotein (P-gp, *ABCB1*) and breast cancer resistance protein (BCRP, *ABCG2*), which are expressed highly in the intestine where these transporters influence FXaI absorption and in the kidneys where they influence renal excretion (105, 106). Concomitant medications that interact with these proteins or functional genetic variants that exist within their respective genes might be associated with increased systemic FXaI exposure and contribute to inter-individual variation in drug concentration. In particular, concomitant use of potent inhibitors and inducers of CYP3A4 such as ketoconazole and rifampin, respectively, have been shown to alter apixaban and rivaroxaban exposure by up to 50% change in the AUC by increasing and decreasing FXaI concentrations, respectively (48, 98, 113, 114). Recent work from Ueshima et al. in a Japanese AF population demonstrated that the *ABCG2* 421A/A genotype was associated with higher apixaban plasma concentrations at trough and reduced clearance, whilst the *CYP3A5*1/*1* genotype was associated with lower concentrations at trough and higher clearance (115, 116). Refer to **Figure 1.5** for the proposed disposition of apixaban and rivaroxaban, as well as **Table 1-3** for a summary of their pharmacokinetic parameters.

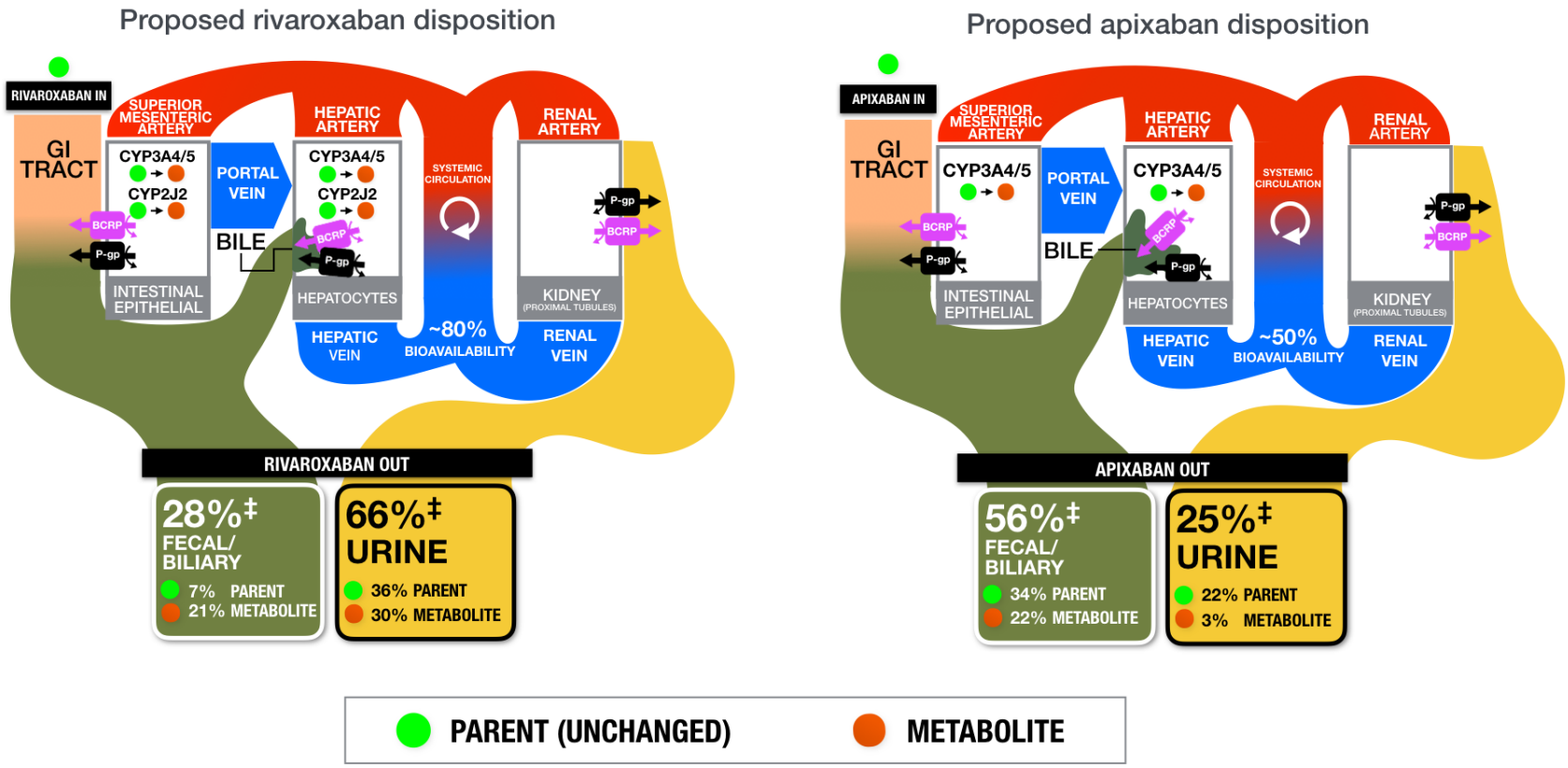

Figure 1-6. Proposed disposition of rivaroxaban and apixaban.

Data represents percent of orally administered radio-labelled dose that is excreted in urine *versus* feces, taken from mass-balance studies carried in healthy human subjects (100, 101). Abbreviations: BCRP, breast cancer resistance protein; CYP, cytochrome P450 enzyme; GI, gastrointestinal tract; P-gp, P-glycoprotein

1.6 Human genetic variation and pharmacogenetics

Sequencing the human genome (117-119) accelerated investigations of the influence of genetic variation on human health and disease. Within the roughly 3.1 billion base pairs of the average human genome, over 12 million common sequence variations (excluding structural variants) (120) have been identified across world populations. These variations can be classified according to their prevalence within a specific population as well as by their nucleotide composition. The latter can be further categorized into structural or single nucleotide variants (

Figure 1-7).

1.6.1 Single nucleotide variants

Single nucleotide variants (SNVs) are sequence variations that occur throughout the genome, in which a single deoxyribonucleic acid (DNA) base pair is substituted for another. They are the most abundant form of genetic variation and based on the frequency of the minor allele in a human population, are referred to as either common or rare SNVs. Common SNVs, also called single nucleotide polymorphisms (SNPs), have a minor allele frequency (MAF) of at least 1%, with most having a frequency greater than 5%. The vast majority of SNVs that are present in an individual are common (121). Rare or novel *de novo* SNVs have a MAF less than 1%, and are sometimes referred to as a mutation, especially when they have a confirmed deleterious effect.

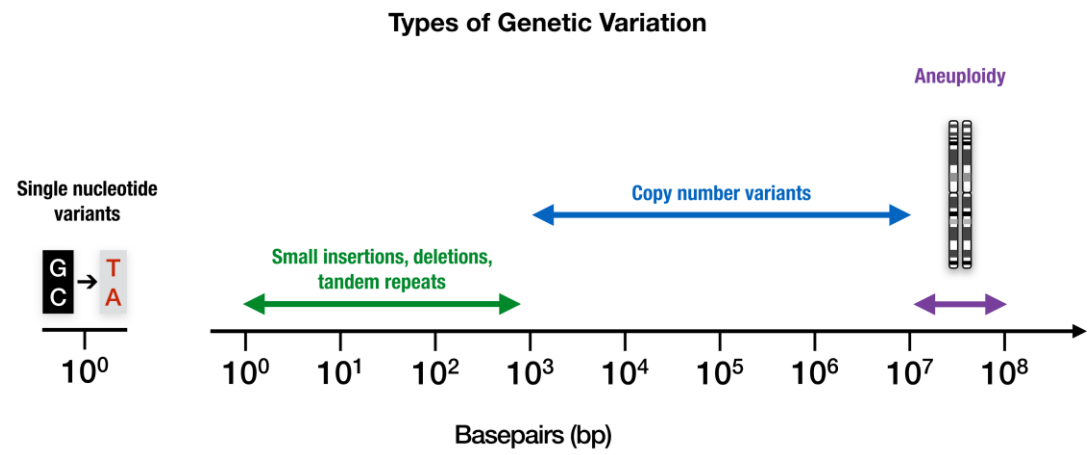

Figure 1-7. Types of genetic variation.

Genetic variation can be classified based on the number of nucleotides that are implicated, which can range from as small as a single nucleotide variant all the way up to a complete loss or gain of a chromosome (aneuploidy).

Aside from allele frequency, SNVs can differ in their location with respect to a gene, and their translated effect at the protein level (**Figure 1-8**). Within the coding region, areas of the genome that undergo transcription and translation, nucleotide substitutions have the potential to alter the amino acid sequence of a polypeptide. A *synonymous* SNV is defined as a nucleotide substitution that creates a codon that codes for the same amino acid. *Nonsynonymous* SNVs occur when a nucleotide substitution creates a codon that either codes for a different amino acid or a premature stop codon. These variants have potential implications on the structure and/or function of a protein. In addition, SNVs can reside within noncoding regions. SNVs that occur within intron-exon boundaries can interrupt the splicing pattern required to form the appropriate messenger ribonucleic acid (mRNA) transcript required for a functional protein. Furthermore, SNVs can occur within regulatory elements (which include promoters, enhancers, repressors) and potentially alter the expression of a gene.

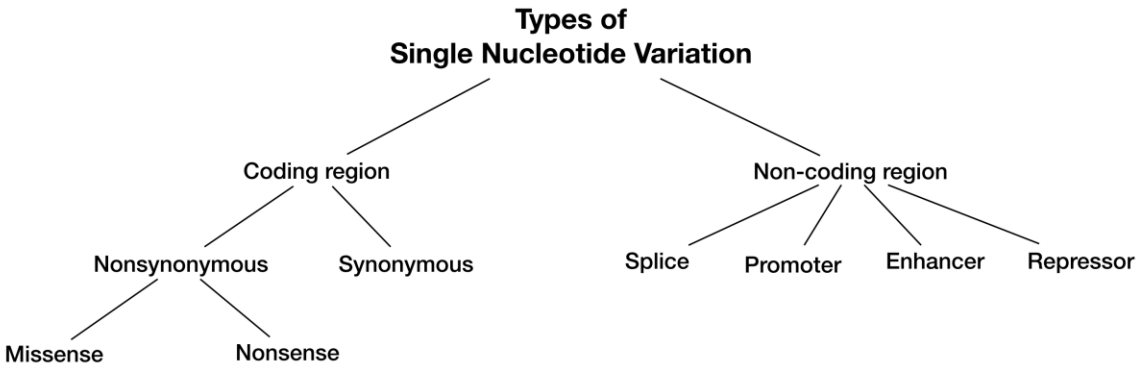

Figure 1-8. Type of single nucleotide variation.

1.6.2 Evolution of pharmacogenetics

In pharmacology, an individual's response to a drug can be considered a phenotype, whether the individual is experiencing toxicity or the desired pharmacological effect. *Pharmacogenetics* is the study of the influence of genetic variation on drug response, typically by way of altering *pharmacokinetics* (absorption, distribution, metabolism, or elimination) or *pharmacodynamics* (target of a drug, or drug sensitivity). Prior to recent advancements, the discoveries that shaped the field of pharmacogenetics came from studying families for patterns of inheritance for specific drug responses, eventually leading to molecular studies that revealed the genetic determinant for that response phenotype (122). Pharmacogenetic research was greatly advanced by the elucidation of the human genome sequence and identification of the common genetic variations within the human population, together with developments in sequencing and genotyping technologies. While previous strategies relied on the candidate-gene approach (pre-selection of genes based on *a priori* knowledge), SNP genotyping arrays have enabled unbiased genome-wide analyses associating SNPs to drug phenotypes through GWA studies. There have been a number of important genotype-phenotype associations identified in pharmacogenetics using GWA studies (123-126). A prominent example of this is the association of common SNVs within the genes, *CYP2C9* and *VKORC1,* with variability in warfarin drug response (73, 74). To date, these arrays have been used to identify several common polymorphisms as genetic factors that influence variability in drug response. Unfortunately, the commonly used genotyping platforms limit the identification of rare or novel SNVs, thus the investigation of rare variations requires more comprehensive sequencing technologies.

1.7 DNA sequencing technologies

DNA sequencing technologies have improved greatly over the past twenty years, reducing the cost and time requirements of large scale sequencing projects. Interestingly, the initial sequencing of the human genome (117) was carried out using a method developed in 1977 (127) by Sanger and his colleagues now known as “Sanger” sequencing. This method was part of a first generation of sequencing technologies, has now been succeeded by a second generation of sequencing technologies. These next generation sequencing (NGS) platforms that drastically improved sequencing cost and time through massive parallelization of sequencing reactions achieved by miniaturizing sequencing reactions and improving detection systems.

1.7.1 Sanger sequencing

In this approach, a single stranded DNA template is replicated several times over using DNA polymerase, an oligonucleotide primer, deoxyribonucleotide-triphosphates (dNTPs), and fluorescently labelled dideoxyribonucleotide-triphosphates (ddNTPs). During DNA synthesis, the incorporation of ddNTPs terminates the elongation of the complementary strand as ddNTPs lack the required 3′OH for elongation. The presence of both ddNTP and dNTP enables the synthesis of complementary DNA strands of varying lengths with one of four fluorescently labelled ddNTPs (ddATP, ddTTP, ddGTP, ddCTP) on their 3′ ends. After several rounds of template DNA extension, the resulting reaction product is injected onto a capillary and separated according to size with the smallest fragments undergoing excitation and detection of fluorescence signal first. This produces four electropherograms that are superimposed to produce the final sequence. The major drawbacks of this method

are the price per base and its inefficiency for large scale sequencing projects. Sanger sequencing of the human genome in the Human Genome Project was a 13-year, $2.7 billion effort.

1.7.2 Illumina sequencing

Today, the *Illumina* sequencer is one of the most commonly used next generation sequencing (NGS) platforms. The methodology involves bridge amplification to produce clusters of clonally enriched template DNA that are then sequenced in a sequencing-by-synthesis manner.

1.7.2.1 Cluster generation

First, template DNA is fragmented and two different sets of adapters are attached to the termini of each fragment. The adapters serve as an annealing site for sequencing primers, as well as a method of attachment to the flow cell (sequencing apparatus). Next, fragments are denatured to form single stranded template DNA that is injected into the flow cell. The flow cell is a glass surface densely coated with different oligonucleotides that are complementary in sequence to the adapters at each end of the template DNA, so that upon injection, the fragments become attached to the surface of the flow cell. Then, a polymerase creates a complement of the hybridized fragment and the original template DNA is washed away. The remaining fragments are then amplified through bridge amplification, transforming single fragments into clonal clusters.

Bridge amplification begins with an annealing step, where the free adapter end tips over and hybridizes to a complementary oligonucleotide on the flow cell forming a bridge.

Next, polymerase chain reaction (PCR) reagents are added to synthesize the complementary strand. Then, the two strands are denatured, which disassociates the bridge. This process of bridge amplification is cycled repeatedly until each cluster contains approximately 1000 clonally amplified fragments, at which point they are ready for sequencing.

1.7.2.2 Sequencing-by-synthesis

The first cycle of sequencing begins with the introduction of DNA polymerase, a primer, and four fluorescently-labelled nucleotides into the flow cell. After primer annealing, a single complementary dNTP is incorporated, and the excess dNTPs are washed away. The fluorescent label also serves to terminate further extension of the sequence, so that only one nucleotide is added at a time. The fluorescent dyes are then excited and an image is taken, capturing an emitted signal for each cluster in the flow cell. The chemical groups containing the fluorescent dye are enzymatically removed recovering the 3′OH group, to allow for the next cycle of dNTPs to be added and imaged. These series of images are then assembled and analyzed by computer software to put together the sequence for the tens of millions of clusters.

1.7.3 Targeted exome sequencing

Although NGS enables the unbiased detection of common, rare, or novel variations, whole genome sequencing is still a costly endeavour. This cost encompasses the price for sequencing as well as data storage and processing. Exome sequencing is a more focused method of sequencing that is limited to the protein coding regions, which comprise

approximately 1% of the human genome (128). These “targeted” exome capture strategies enable deep sequencing of a relatively small number of selected genes of interest (cumulatively less than 1,000,000 bp), decreasing error rates and uncertainly in genotype calling that are frequently associated with short-read NGS data (129). Fundamentally, targeted exome NGS (**Figure 1-9**) requires the capture and enrichment of genomic regions of interest before sequencing. The target-enrichment strategies that can be used include PCR-based, molecular inversion probe (MIP)-based, or hybrid oligonucleotide capture-based approaches, all of which can vary in their performance relative to one another (130). The possibility of using NGS to sequence genes pertinent to drug disposition and drug response is of particular relevance in the fields of pharmacogenetic research and clinical genotyping to implement personalized genotype-based therapies

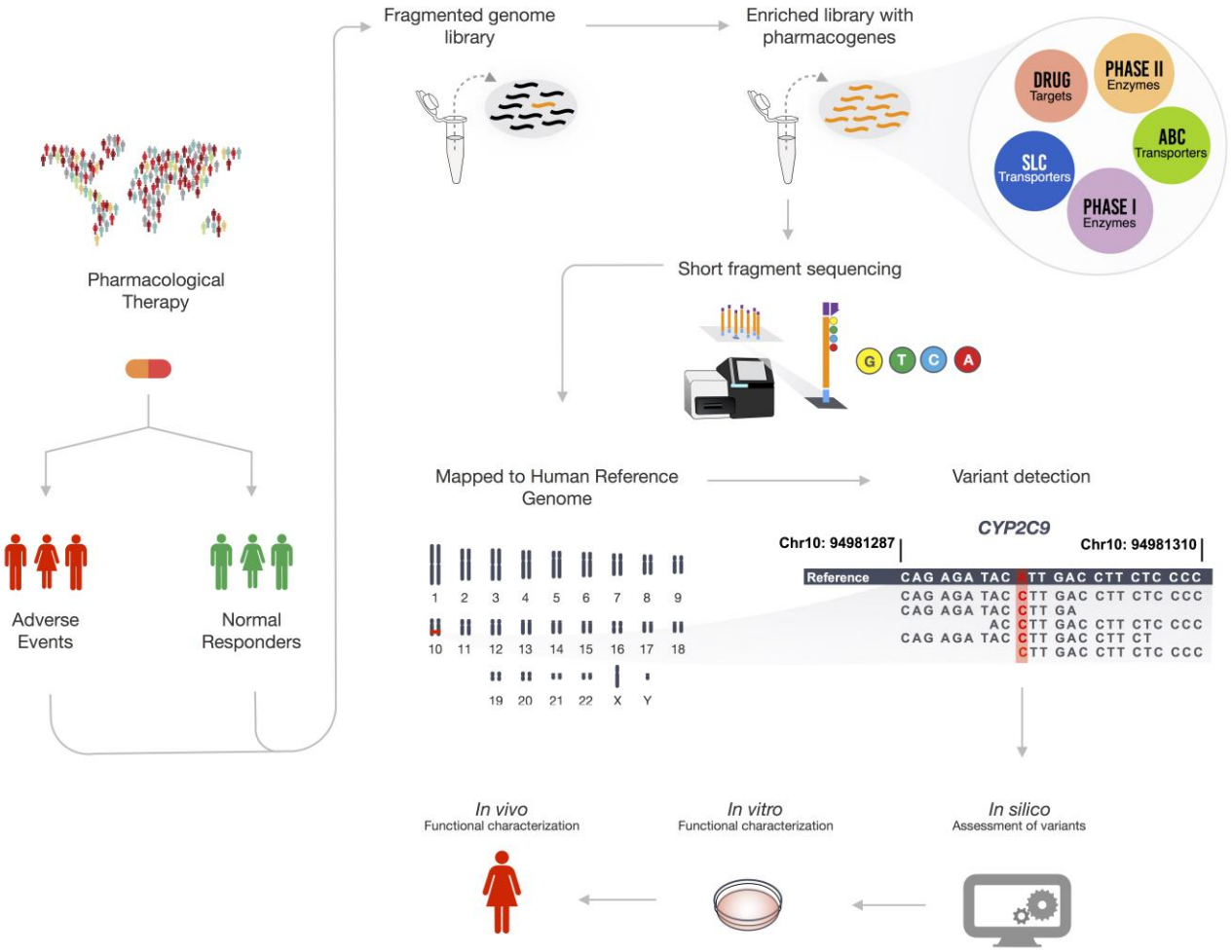

Figure 1-9. Targeted next generation sequencing workflow.

1.8 Summary

Factor Xa inhibitors (FXaIs), rivaroxaban and apixaban, represent a new class of oral anticoagulants that are a safe and efficacious alternative to warfarin therapy for stroke prevention in patients with AF. Given their mechanism of action, FXaI pharmacological effect can be inferred based on the observed plasma drug concentration. Consequently, prolonged periods of elevated FXaI concentration are associated with increased risk for major bleeding. Currently, there is a paucity of data relating to observed interpatient variation in FXaI plasma concentrations in the post-market clinical setting. The patient population that take FXaIs in the post-market setting may vary greatly with their co-morbidities and extensive co-medications from those within clinical trials. Therefore, the systemic FXaI exposures achieved in these individuals may vary greater than those observed in clinical trials, and this interpatient variation is likely driven by patient-specific factors that comprise an individual's physiology, disease-state, environment (e.g. co-medications, food), as well as genetics. Renal impairment, age, sex, extremes in weight, as well as common single nucleotide variants (SNVs) in metabolizing enzymes and transporters that are responsible for apixaban and rivaroxaban disposition have been indicated to alter the elimination of FXaIs from the body, and are predictive of systemic apixaban exposure. Similarly, endogenous biomarkers of FXaI metabolism may serve as an additional predictor of drug exposure. While previous research surrounding the influence of genetics on drug exposure has been focused on evaluating common functional variation, more recent evidence suggest rare SNVs in drug processing genes may also significantly contribute to interpatient differences in drug disposition beyond established common genetic predictors (131-133). Thus it is intriguing to speculate that AF patients

who demonstrate unexpectedly high or low FXaI plasma concentration may harbor rare or patient-specific SNVs that result in aberrant function or expression of their FXaI metabolizing enzyme or transporter, consequently impacting their drug exposure.

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2 Specific Aims and Hypotheses

2.1 Specific aim 1

1. **To measure the extent of the variability in plasma apixaban and rivaroxaban concentration within atrial fibrillation (AF) patients in a routine care setting.**

Factor Xa inhibitors (FXaIs), rivaroxaban and apixaban, represent a new class of oral anticoagulants that are widely prescribed as an alternative to warfarin therapy for stroke prevention in patients with AF. An important advantage of these drugs is that routine monitoring of anticoagulation response is not necessary. Nevertheless, because of their mechanism of action a FXaI anticoagulation effect can be inferred based on the observed plasma concentration, with prolonged periods of elevated FXaI concentration associated with increased risk for major bleeding. Currently, there is a paucity of data relating to observed interpatient variation in FXaI plasma concentrations in the post-market clinical setting. Given that the patient population that take FXaIs in the post-market setting may vary greatly with their co-morbidities and extensive co-medications from those within clinical trials, the systemic FXaI exposures achieved in these individuals may vary greatly as well.

We hypothesize that the variation in the apixaban and rivaroxaban concentration within the routine care setting is greater than the variation observed during clinical trials.

To test this hypothesis, we determined rivaroxaban and apixaban plasma concentrations in a cohort of 243 AF patients during routine clinic visits. As described in Chapter 4, in our

routine-care setting rivaroxaban and apixaban plasma concentrations tended to be more variable than those observed in clinical trials. Approximately 12% of patients receiving rivaroxaban and 13% of patients receiving apixaban exceeded the 95th percentile for the predicted maximum FXaI plasma concentration (C_{max}) observed in clinical trials. Overall, identification of additional clinical and molecular determinants that more fully predict patients at risk for excessively high or low FXaI concentrations may enable a more precise FXaI dosing regimen for the individual patient.

2.2 Specific aim 2

1. **To identify predictors of variability in FXaI plasma concentration among apixaban-treated AF patients using clinical variables, common genetic variation, and an endogenous biomarker for apixaban metabolism.**

Apixaban undergoes multiple pathways of elimination that include both metabolism, and excretion, though renal, intestinal, and biliary mechanisms (1, 2). Factors that influence these elimination pathways such as renal function, age, sex, weight, and common genetic polymorphisms have been demonstrated to alter the pharmacokinetics of apixaban in subjects, and are predictive of systemic apixaban exposure (3-6). Similarly, identifying a factor that can be used as a measure of apixaban metabolism (endogenous biomarker) may serve as an additional predictor of variability in apixaban exposure, especially given that roughly one third of orally administered apixaban is excreted from the body in metabolite form. Hepatic and intestinal cytochrome P450 3A enzymes (CYP3A4/5) are responsible for the phase I metabolism (oxidation) of apixaban (7, 8). In humans, 4β-hydroxycholesterol (4β-OHC) is an endogenous oxysterol, present in the blood, that is

created through the conversion of cholesterol by CYP3A enzymes (9), and is recognized as an emerging biomarker of CYP3A activity. Therefore, by examining 4β-OHC as a marker for apixaban metabolism, together with factors that are known to impact apixaban exposure (renal function, age, sex, weight, and common genetic variation), we are likely able to better explain the variation in concentration observed within our apixaban-treated cohort.

We hypothesize that CYP3A activity, as measured by 4β-OHC concentration is a predictor of apixaban plasma concentration, and together with known clinical and genetic determinants of apixaban exposure will better explain interpatient variation in FXaI concentration compared to clinical and genetic factors alone.

To test this hypothesis, we determined 4β-OHC plasma concentrations in 136 characterized and genotyped AF patients. As described in Chapter Four, we found a weak but statistically significant correlation between plasma concentrations of apixaban and 4β-OHC, with higher 4β-OHC concentrations (higher CYP3A4 activity) associated with lower apixaban concentrations. Regression analysis demonstrated that 4β-OHC concentration was an independent predictor of apixaban concentration in our cohort, along with age, female, serum creatinine, and amiodarone use. Weight, diltiazem use, as well as *CYP3A4*22*, CYP3A5*3, *ABCB1* c.3435C>T, or *ABCG2* c.421C>A carrier status were not significant predictors of apixaban plasma concentration in our cohort.

2.3 Specific aim 3

1. **To develop a targeted next generation sequencing (NGS) approach for the detection of genetic variation in pharmacogenes pertinent to drug disposition and response.**
2. **To apply this method for single nucleotide variant (SNV) discovery in a small cohort of apixaban-treated AF patients with aberrantly high drug plasma concentrations.**

In Chapter Four, we noted that known common genetic variation in metabolizing enzymes and efflux transporters (*CYP3A4*22*, *CYP3A5**3, *ABCB1* c.3435C>T, or *ABCG2* c.421C>A) were not significant predictors of interpatient variation in apixaban concentration within our AF cohort. Furthermore, a large proportion (~72%) of this interpatient variation remained unaccounted for within our regression analysis. Therefore, we proposed that rare or patient-specific single nucleotide variations (SNV) within certain individuals are likely contributing to this unexplained variation. While previous pharmacogenetic research has focused on evaluating common functional variation (minor allele frequency, [MAF] >5%), more recent large-scale whole genome or exome sequencing studies have revealed that humans harbor a large number of rare (MAF <1%), potentially deleterious variants in many of these pharmacogenes (10, 11). Further studies support that rare SNVs in drug processing or drug target genes significantly contribute to interpatient differences in drug disposition and response beyond established common genetic predictors as shown for *CYP2C9* and warfarin dose requirement (12, 13) and *SLCO1B1* and methotrexate clearance and toxicity (14). Targeted next-generation

sequencing (NGS) represents a new time- and cost-effective technology for detecting common and rare genetic variation, allowing for DNA enrichment of entire exomes or coding exons of select genes coupled with massive parallel sequencing of the enriched product. Through the application of this method, particularly, in those apixaban-treated AF subjects who demonstrated unexpectedly high drug plasma concentrations, we would be able to better identify those individuals with rare or patient-specific SNVs that result in aberrant function or expression of their CYP enzyme or transporter, consequently impacting their apixaban exposure.

We hypothesize apixaban-treated AF subjects with aberrantly high apixaban plasma concentrations harbor rare or patient-specific functional SNVs within genes involved in the metabolism and transport of apixaban.

To test this hypothesis, we generated a custom panel of capture probes to enrich a target sequence of 422 kb of coding region in 100 pharmacogenes, including seven genes that are relevant to apixaban disposition. NGS was carried out in 245 subjects (n=48/245 validation cohort; n = 12/245 apixaban-treated AF cohort). As described in Chapter five we generated a sufficient depth-of-coverage (DOC) > 30 × for 97.4% of the target sequence, with a total of 1089 variants detected in 245 subjects. Selected SNVs (32 common and 5 rare or novel) identified through NGS were found to be concordant with TaqMan genotyping and Sanger sequencing results. Importantly, 60% of total variants were rare or previously unreported, with a greater proportion of rare or novel SNVs predicted to alter protein function compared to common SNVs. Each subject carried at least one predicted functional variant

on both alleles across our target sequence, and 161 subjects (66%) were homozygous carriers for at least one clinically actionable variant. More specifically, of the apixaban-treated AF subjects that were sequenced, our exploratory analysis did not reveal potentially deleterious SNV(s) within the selected candidate genes that may further explain their observed elevated apixaban plasma concentration.

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3 Interpatient Variation in Rivaroxaban and Apixaban Plasma Concentrations in Routine Care [1]

1. Reprinted with permission from Gulilat M, Tang A, Gryn SE, Leong-Sit P, Skanes AC, Alfonsi JE, et al. Interpatient Variation in Rivaroxaban and Apixaban Plasma Concentrations in Routine Care. The Canadian journal of cardiology. 2017;33(8):1036-43.

3.1 Introduction

Direct-acting oral anticoagulants (DOACs) that target key rate-limiting enzymes in the coagulation cascade are rapidly replacing warfarin as therapeutic agents of choice for stroke prevention in the setting of atrial fibrillation (AF), as well as prevention or treatment of venous thromboembolism (1-8). A key perceived advantage associated with the use of DOACs is the relative ease in dosing and no requirement for routine monitoring of anticoagulation response, such as INR (9).

Rivaroxaban and apixaban, both Factor Xa (FXa) inhibitors, elicit their pharmacological effect in a concentration-dependent manner (10-14). Preclinical studies measuring isolated human FXa enzyme activity over varying drug concentrations demonstrated an inhibitory effect proportional to spiked plasma drug concentrations (2, 15). During Phase I (in-human) studies, these findings translated into prolonged clotting times, with the anticoagulation effect directly relating to plasma concentration (10-14). As such, there is a particular concern for increased bleeding risk with higher plasma concentrations which has spurred the development of reversing agent for use in the setting of major bleeding events (16-18).

The European Medicines Agency (EMA) and US Federal Drug Administration (FDA), have shown interest in defining an acceptable therapeutic index that is clinically impactful and actionable (16, 18, 19). To address the gap in knowledge of DOAC drug concentrations in patients during routine care, we aimed to assess DOAC concentrations in patients with existing AF who were prescribed rivaroxaban or apixaban as a part of standard care.

3.2 Materials and Methods

3.2.1 Study design and participants

This prospective cohort study was carried out at the London Health Sciences Center, University Hospital in London, Ontario. Patients with existing atrial fibrillation (AF) prescribed rivaroxaban or apixaban at Cardiology or Anticoagulation Clinics were invited to participate. Inclusion and exclusion criteria are outlined in **Figure 3-1**. The study protocol was approved by the Health Sciences Research Ethics Board of the University of Western Ontario (London, Canada), and written informed consent was obtained. The study was conducted over a 3-year period (February 2013 and January 2016).

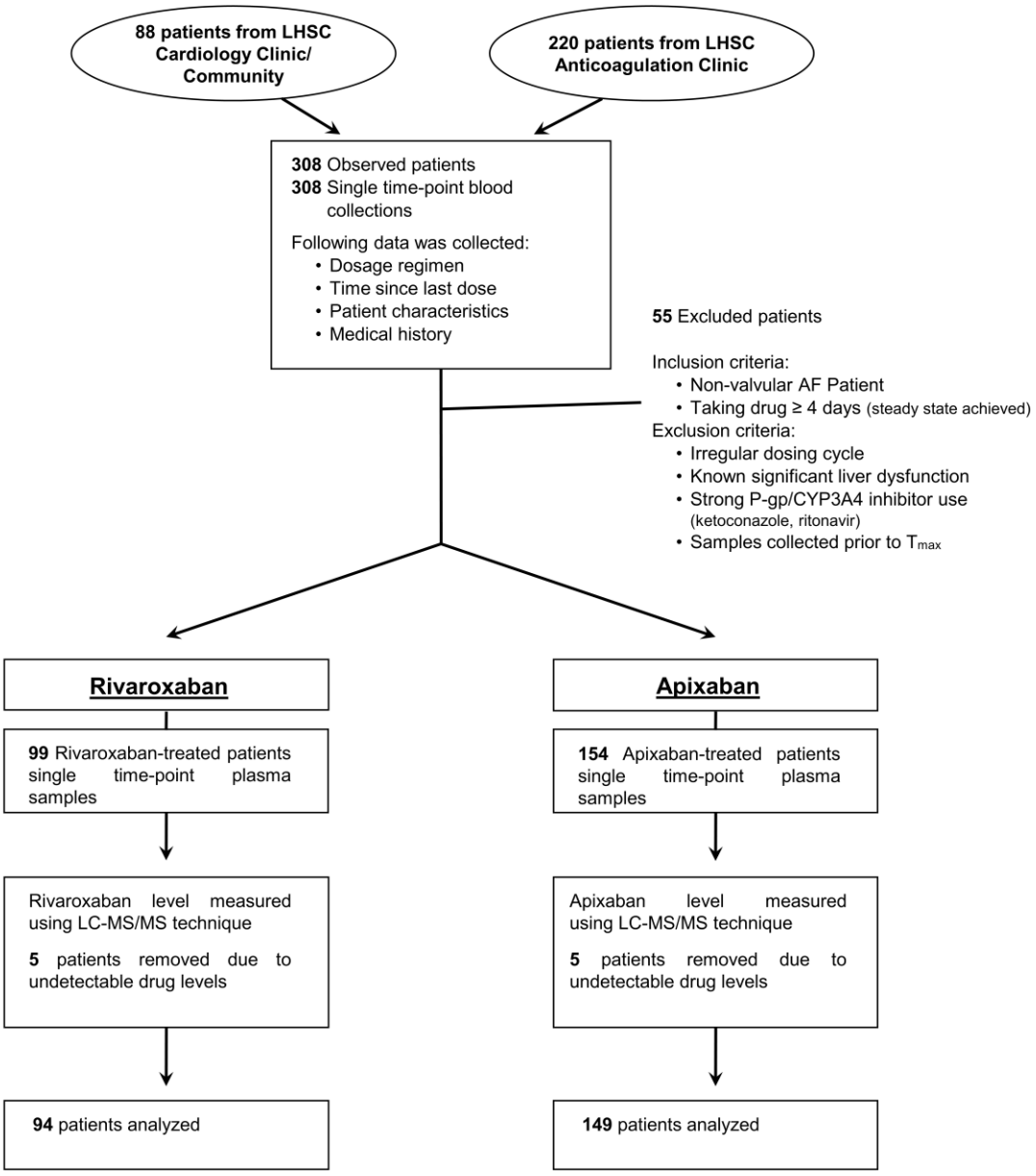

Figure 3-1. Patient flow diagram.

Enrolled patients must have been on the same dose for ≥ 4 days at the recommended dosing cycle for rivaroxaban (once daily) and apixaban (twice daily), with only those samples collected >2 (rivaroxaban) and >3 (apixaban) hours post dose to be considered in the drug concentration analysis. Abbreviations: LHSC, London Health Sciences Center; Tmax = time of maximum concentration.

3.2.2 Clinical data collection

Patient age, sex, weight, and height was collected at the time of blood sampling, along with their most recent serum creatinine concentration to the time of draw. Renal function is known to have a greater effect on observed rivaroxaban plasma concentration when compared to apixaban (20). Renal function was monitored by determining the estimated creatinine clearance (eCrCl) using the Cockcroft-Gault formula. Rivaroxaban and apixaban are both reported substrates of the drug efflux transporter P-glycoprotein (P-gp), and drug metabolizing cytochrome P450 (CYP) enzyme CYP3A4 (21-24). Accordingly, concomitant use of moderate (amiodarone, diltiazem, fluconazole, verapamil) to strong (clarithromycin, ketoconazole, ritonavir) P-gp/CYP3A4 inhibitors(25), and P-gp/CYP3A4 inducers(25) (carbamazepine, phenytoin, phenobarbital rifampin) were documented at the time of sampling.

3.2.3 Sample collection, processing, and storage

Blood samples were obtained from each patient while taking their prescribed dose of rivaroxaban or apixaban. Date and time of last dose and blood collection was recorded and time since last dose (hours) was subsequently calculated. Blood samples were immediately placed at 4°C, prior to centrifugation at 2000 *g* for 10 minutes for plasma isolation. Plasma samples were stored at -80°C until further analysis.

3.2.4 Determination of rivaroxaban and apixaban plasma concentration.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays for rivaroxaban and apixaban were developed and validated using modified protocols (26, 27). Rivaroxaban, rivaroxaban-D4, and apixaban were obtained from Toronto Research Chemicals (Toronto, Canada) and dissolved in dimethyl sulfoxide at 1mg/ml. [^{13}C, $^{2}H_7$]-Apixaban (Alsachim, Illkirch-Graffenstaden, France) was dissolved in methanol at 1mg/ml. Calibration standards (0, 5, 10, 25, 50, 100, 150, 250, 500, 750, 1000 ng/mL) and quality controls (QCs) (25, 250, 1000 ng/ml) were prepared in drug-naïve, non-filtered (K_2-EDTA) human plasma (Bioreclamation IVT, Maryland, USA). QCs were used to calculate the precision and accuracy of the assay according to standards of the FDA Bioanalytical Method Validation for chromatography (28). Standard or QC plasma (100 µl), and patient plasma (50 µl) were respectively dissolved in 300 µl and 150 µl of acetonitrile spiked with rivaroxaban-D4 or [^{13}C, $^{2}H_7$]-apixaban as an internal standard. Samples were kept at ambient temperature for 5 minutes followed by centrifugation at 13,500 rpm for 20 min at 4°C. A volume of 100 µl of the supernatant was subsequently diluted with 75 µl of 0.1% formic acid in H_2O and transferred to glass auto-sampler vials. Chromatographic separation was performed using an Agilent1200 G1312 Binary Pump (Agilent Technologies, Santa Clara, USA) and a Hypersil GOLDTM C18 (50mm x 3mm ID, 5 µm particle size) column (Thermo Scientific, San Jose, USA), with an injection volume of 40 µl. Mobile phases used for gradient elution were 0.1% formic acid in water and acetonitrile. The mass analysis was performed on a TSQ Vantage triple-quadrupole mass spectrometer (Thermo Scientific). The following mass transitions were monitored in positive mode for quantification: apixaban, 460.1⟶ 443.3 m/z; [^{13}C, $^{2}H_7$]-apixaban, 468.1⟶ 451.4 m/z;

rivaroxaban, 436.4⟶144.9 m/z; and rivaroxaban-D4, 440.8⟶144.9 m/z. Assay performance across the 25, 250, and 1000 ng/ml QCs were as follows: inter-day bias (accuracy) and precision were respectively less than or equal to 5.2% and 4.2% for rivaroxaban, and 1.5% and 8.5% for apixaban; intra-day bias and precision were respectively less than or equal to 5.3% and 3.5% for rivaroxaban, and 1.3% and 5.1% for apixaban.

3.2.5 Reference rivaroxaban and apixaban clinical trials population pharmacokinetics data.

To compare the distribution of measured single time-point plasma concentrations in relation to clinical trials, rivaroxaban and apixaban treated AF population concentration data were sourced from Girgis et al (29) and the *Eliquis*® (apixaban) product monograph (30) respectively. Rivaroxaban measured concentrations were compared to the population concentration-time profile sourced from Girgis et al (presented in **Figure 3-2**, as median and 5^{th}-95^{th} percentile). Maximum and minimum concentration values were taken directly from the concentration-time profile. This simulated concentration-time profile of the 20 mg once daily rivaroxaban dose was obtained through population pharmacokinetic modelling of sparsely sampled plasma concentration data from phase III (ROCKET AF) rivaroxaban-treated atrial fibrillation patients (29). For apixaban, there are currently no available published pharmacokinetic data that describe the entire time course of apixaban concentrations specific to atrial fibrillation patients. Therefore, measured concentrations were compared in relation to the maximum (C_{max}) and minimum (C_{min}) plasma concentration values over the dosing interval (presented in Figure 3-3, as median and 5th-95th percentile) provided in the *Eliquis*® (apixaban) product monograph for nonvalvular

atrial fibrillation patients prescribed apixaban 5 mg twice daily. Specifically, we assessed whether the measured apixaban concentrations fell within the range of 41 ng/ml to 321 ng/ml, representing the lower limit for Cmin (5th percentile) to the upper limit for Cmax (95th percentile) respectively (30).

3.2.6 Statistical analysis

Analysis was performed using the statistical software, SPSS (Armonk, USA) and GraphPad Prism 6 (La Jolla, USA).

3.3 Results

3.3.1 Patient characteristics

We analyzed DOAC concentration data from 243 AF patients (rivaroxaban, n=94; apixaban, n=149) with subject characteristics summarized in **Table 3-1**. Compared to clinical trials, our cohort had a higher proportion of females as well as higher rates of concomitant moderate P-gp/CYP3A4 inhibitor use (amiodarone, verapamil, diltiazem). For apixaban, our cohort had a higher percentage of patients who were ≥75 years of age when compared to patients enrolled in the ARISTOTLE and AVERROES clinical trials (50.3% compared to 31.2% and 33.8%, respectively).

Table 3-1. Study population characteristics in relation to published trials.

Characteristic	Current Study		Published Trials		
			ROCKET AF (5)	ARISTOTLE (31)	AVERROES(6)
Drug	Rivaroxaban	Apixaban	Rivaroxaban	Apixaban	Apixaban
Subjects, n	94	149	7131	9120	2808
Age, median (IQR) or mean (SD)	68 (60-78)	75 (66-82)	73 (65-78)	70 (63-76)	70 (9)
Age, ≥65 years, (%)	60.6	79.2	76.9 (32)	69.9 (33)	69.3
Age, ≥75 years, (%)	30.9	50.3	44.0 (34)	31.2	33.8
Sex, Female (%)	45.7	47.7	39.7	35.3	41.5
BMI, kg/m2, median (IQR) or mean (SD)	29 (24-34)	29 (25-34)	28 (25-32)	NR	28 (5)
Weight, kg, median (IQR)	82 (70-99)	83 (70-97)	NR	82 (70-95)	NR
CHADS2, mean (SD)	2.0 (1.3)	2.6 (1.3)	3.5 (1.0)	2.1 (1.1)	2.0 (1.1)
Rivaroxaban Dose					
15 mg daily, (%)	25.5	-	20.7 (35)	-	-
20 mg daily, (%)	74.5	-	79.2 (35)	-	-
Apixaban Dose					
2.5 mg twice daily, (%)	-	38.3	-	4.7	6.4
5 mg twice daily, (%)	-	61.7	-	95.3	93.6

Concomitant moderate P-gp and CYP3A4 Inhibitor use, (%)	30.9	30.8	18.2 (36)	-	-
Amiodarone, (%)	11.3	13.4	NR	11.1	11.0
Diltiazem and Verapamil, (%)	19.6	17.4	NR	NR	9.0
Concomitant P-gp and CYP3A4 Inducer use, (%)	5.2	2.8	NR	NR	NR
Phenobarbital, (%)	0.0	0.7	NR	NR	NR
Phenytoin, (%)	3.1	0.7	NR	NR	NR
Carbamazepine, (%)	2.1	0.7	NR	NR	NR
Rifampicin, (%)	0.0	0.7	NR	NR	NR
Hours post dose, mean (min-max)	11.8 (2-23.5)	6.2 (3-11.9)	-	-	-
Renal function, creatinine clearance*			7111 (32)	9120 (33)	2798(33)
subjects, n	94	149			
Normal (>80 ml/min), (%)	52.1	37.6	32.2 (32)	41.2 (33)	34.0 (33)
Mild (50-79 ml/min), (%)	24.5	32.9	46.6 (32)	41.9 (33)	38.2 (33)
Moderate (30-49 ml/min), (%)	17.0	21.5	21.0 (32)	15.0 (33)	17.5 (33)
Severe (15-30 ml/min), (%)	2.1	3.3	NR	1.5 (33)	2.0 (33)
Not Reported, (%)	4.3	4.7	0.2 (32)	0.4 (33)	8.3 (33)
Serum Creatinine, µmol/L, median (IQR)	84 (69-98)	91 (72-111)	NR	NR	NR

Abbreviations: BMI, body mass index; IQR, interquartile range; NR, not reported; SD, standard deviation; Data represented as: percent of cohort (%), median (IQR, 25th and 75th percentile), mean (±SD); *Creatinine clearance estimated using Cockcroft-Gault formula for patients with available serum creatinine.

3.3.2 Rivaroxaban plasma concentrations

We observed approximately 60-fold variation, based on minimum to maximum plasma concentration across the dosing interval, in patients prescribed rivaroxaban 20 mg daily (n=70) and 15 mg daily (n=24). When the observed concentrations in this cohort of patients were plotted in relation to AF population concentration-time profiles assessed during clinical trials (See Methods, for **Figure 3-2** reference details)(29), 40.4% (n=38) of rivaroxaban patient values were outside the predicted 5-95th population percentile for concentrations (**Figure 3-2**). Of the 38 patients outside the predicted concentration range, 21 were above the 95th percentile while 17 were below the 5th percentile. Eleven (11.7%) of measured concentrations across the dosing interval exceeded 363.9 ng/ml, which is the estimated upper limit (95th percentile) of Cmax for an AF patient taking rivaroxaban 20 mg once daily at steady-state (29). Rivaroxaban plasma concentrations were also plotted according to those above or below the estimated creatinine clearance threshold used for dose selection (50 ml/min) (**Figure 3-2B**).

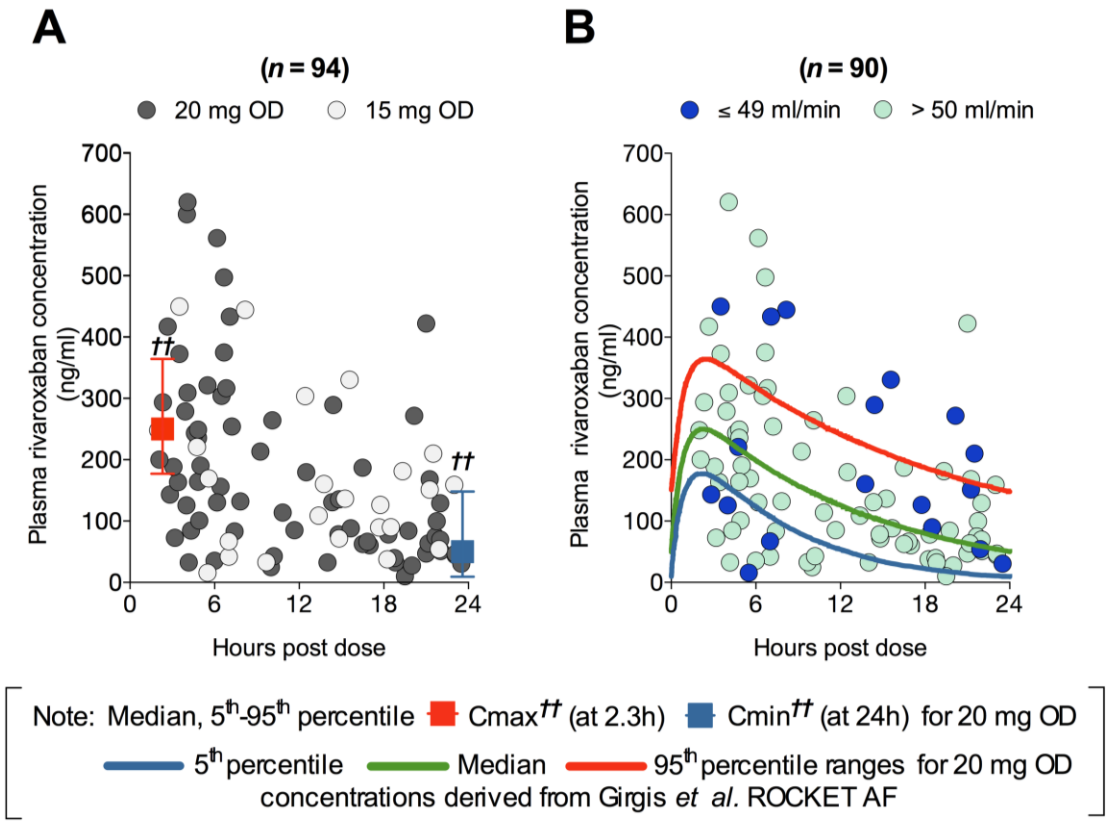

Figure 3-2. Rivaroxaban plasma concentration in AF patients within routine care.

Observed plasma rivaroxaban concentration in relation to dose (A) and criteria required for appropriate dosing: estimated creatinine clearance (B), superimposed on to clinical trial pharmacokinetic population data for atrial fibrillation (AF) patients (See Methods). Estimated creatinine clearance calculated using Cockcroft-Gault's formula, patients missing serum creatinine data were excluded (n=4, panel B). Abbreviations: Cmax maximum concentration; Cmin, minimum concentration.

3.3.3 Apixaban plasma concentrations

A 50-fold variation (minimum to maximum) was observed in measured plasma concentrations for both apixaban dosing regimens: 5 mg twice daily (n = 92) or 2.5 mg twice daily (n = 57). Of the combined 149 patients, 24 (16.1%) attained concentrations outside the maximum (Cmax, 95th percentile) and minimum (Cmin, 5th percentile) plasma concentration values reported in the *Eliquis* product monograph for AF patients taking 5 mg twice daily (**Figure 3-3A**). More specifically, 19 *versus* 5 patients had concentrations above and below the reported Cmax and Cmin, respectively.

The recommended apixaban dose for stroke prevention in AF patients is 5 mg twice daily, or the reduced 2.5 mg twice daily dose in those fulfilling 2 of following 3 clinical criteria: age ≥ 80 years, serum creatinine ≥133 μmol/L, and weight ≤60 kg. The observed apixaban concentrations were also plotted according to these clinical criteria (**Figure 3-3B-D**). Plasma concentrations of patients taking 5 mg *versus* 2.5 mg twice daily (220.6 ng/ml compared to 173.3 ng/ml) were similar with only a modest 1.27-fold difference between the mean measured apixaban concentrations.

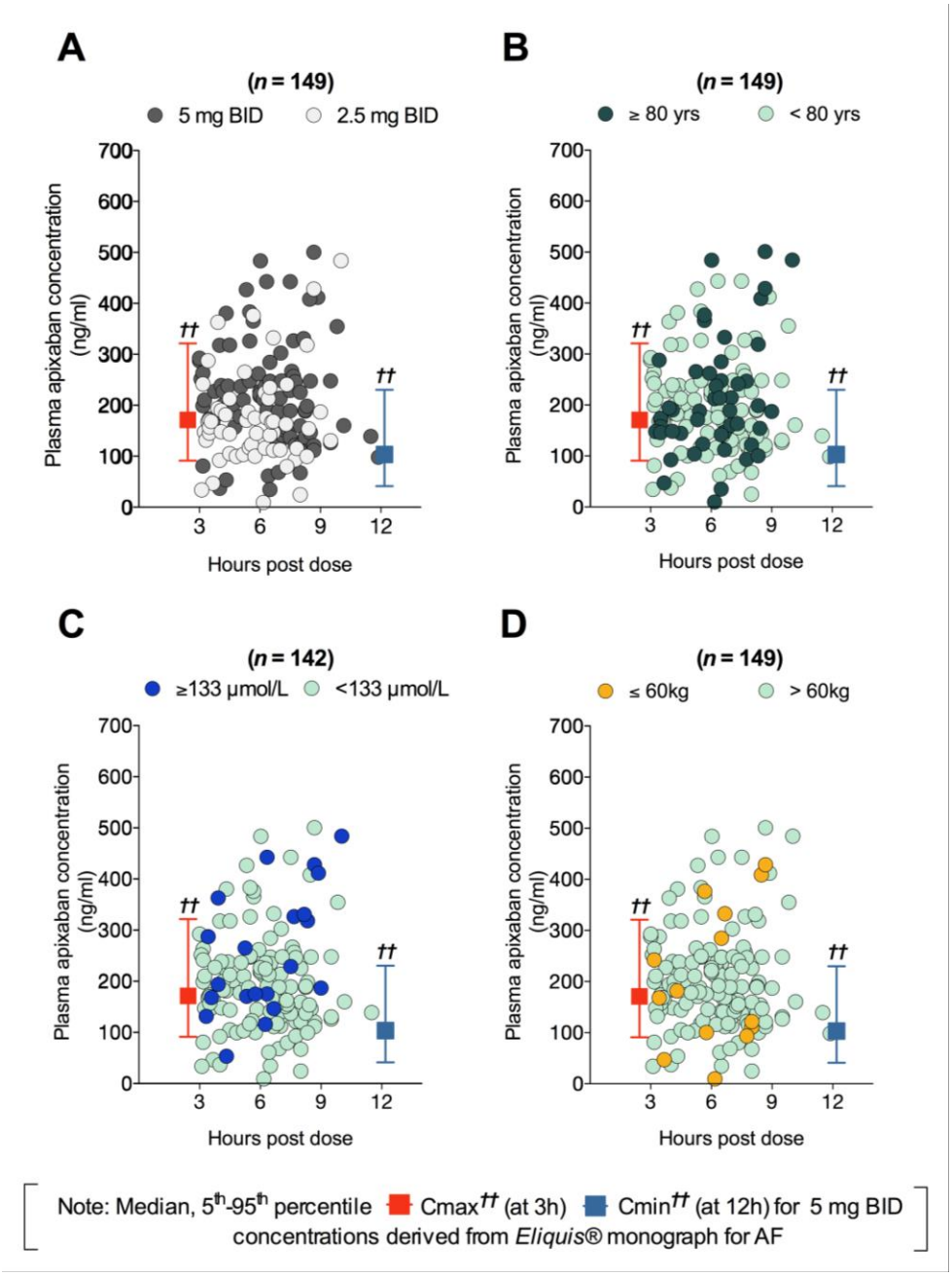

Figure 3-3. Apixaban plasma concentration in AF patients within routine care

Observed plasma apixaban concentration in relation to dose (A), and criteria required for appropriate dosing: age (B), serum creatinine (C), and weight (D). Measured concentration compared to predicted maximum (Cmax) and minimum (Cmin) plasma concentrations values for 5 mg twice daily dose, as noted in the apixaban product monograph for atrial fibrillation (AF) patients. Patients missing serum creatinine data were excluded (n=7, panel C).

3.3.4 Concomitant P-glycoprotein and CYP3A4 inhibitors and inducers

Currently there are no recommendations regarding concomitant use of moderate P-gp/CYP3A4 inhibitors such as amiodarone and diltiazem. In our patients, these agents were prescribed in 30% of our cohort. We compared DOAC plasma concentrations of concomitant amiodarone and diltiazem users to non-users. There was a lack of relationship between moderate inhibitor use and DOAC plasma concentrations, however, this study was not well powered to determine the effect of moderate inhibitor use on DOAC plasma concentrations. We were also unable to assess the effect of P-gp/CYP3A4 inducing medications on DOAC concentrations as few patients were co-prescribed these medications.

3.4 Discussion

3.4.1 Study Summary

This study examined the extent of interpatient variation in plasma DOAC concentrations in AF patients in a routine clinical care setting, prescribed rivaroxaban or apixaban in comparison to that observed in clinical trials. Plasma concentrations of both rivaroxaban and apixaban exhibited marked interpatient variability in that 40% of rivaroxaban patients were outside the 5-95th population percentile ranges for plasma concentrations observed in clinical trials, of which 11.7% exceeded the upper limit (95th percentile) for C_{max} (29). Similarly for apixaban, 12.8% of our patients were above the 95th percentile for Cmax,

whereas 3.4% were below the 5th percentile for C_{min}, as outlined in the apixaban (Eliquis) product monograph (30).

3.4.2 Potential clinical relevance of our findings

Currently, there are no approved blood tests for anticoagulation response to monitor DOAC therapy. Although the attained plasma concentration of DOACs mirror their inhibitory effects (whether thrombin or FXa) on their target (2, 10, 12, 15, 37, 38) the utility of a pharmacokinetic-based approach to improving DOAC therapy is dependent on the association between drug exposure and clinical response. The potential relationship between FXa inhibitor circulating concentration and harm (major bleeding) *versus* efficacy (stroke prevention in AF, venous thrombotic embolism (VTE) in total knee/hip replacement) was noted in the FDA's Clinical Pharmacology and Biopharmaceutics Reviews documents (21, 39). In addition, there is sufficient evidence to suggest the use of ketoconazole, a potent P-gp and CYP3A4 inhibitor, increases DOAC exposure by approximately 2-fold (40, 41). Due to the associated increased bleeding risk, ketoconazole is contraindicated in patients taking rivaroxaban or apixaban (30, 42).

However, at the present time, the precise DOAC concentration thresholds that accurately predict for over- or under-anticoagulation have not been established. Additional studies involving therapeutic drug monitoring (TDM) may help to establish a therapeutic index. Ideally, such a therapeutic index will then define an optimal plasma concentration range that provides greatest benefit, that of stroke prevention for AF patients, while minimizing bleeding risk. We note for the latest DOAC to attain regulatory approval, edoxaban, a detailed assessment of edoxaban exposure *vs* bleeding and stroke or systemic

embolic event risk was published as part of the ENGAGE AF-TIMI 48 trial. Interestingly, the slope for bleeding risk was steeper, relative to the slope for stroke prevention, particularly when the trough concentration exceeded 100 ng/ml (43).

3.4.3 Limitations

This is an observational study of patients who were recruited during their routine care visits. As such, this limits our ability to account for the effect of adherence as well as inaccurate self-reporting of time of last-dose on the drug concentration. It remains possible that unreported missed doses or drug intake without food (rivaroxaban only) may explain some of the inter-patient variability especially in patients with very low plasma concentrations. Finally, this study lacks adequate sample size to assess clinically relevant outcomes, such as major bleeding, stroke, or death. Prospective studies in routine care patients that focus on attained DOAC concentration in relation to outcomes are needed.

3.4.4 Conclusion

In this study, we demonstrate variation in DOAC plasma concentrations in routine care are greater than that observed in clinical trials. Therapeutic drug monitoring may be helpful in DOAC dosing and selection for a subset of patients with variable renal function or requiring concomitant medications that can alter DOAC disposition. Overall, DOACs represent an important new class of medications which are widely prescribed as an alternative to warfarin therapy, and increasingly recommended as the initial oral anticoagulant in the setting of AF with risk factors for stroke (8). Additional research focused on the association of clinical and genetic variables to DOAC concentration and clinical outcomes are needed.

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4 Clinical and molecular determinants of apixaban plasma concentration in routine care

Gulilat M, Pananos AD, Lizotte DJ, Teft WA, Schwarz UI, Tirona RG, Kim RB. Clinical and molecular determinants of apixaban plasma concentration in routine care. Manuscript in preparation, 2018.

4.1 Introduction

Factor Xa inhibitor, apixaban, belongs to an increasingly prescribed class of direct-acting oral anticoagulants (1, 2) used for prevention of stroke (3-5), and venous thromboembolisms (VTE) (6, 7). Under the setting of stroke prevention in patients with atrial fibrillation (AF) the recommended dosage of apixaban is 5 mg taken orally twice daily, or 2.5-mg twice daily dose for those fulfilling at least two of the following clinical criteria: age ≥ 80 years, body weight ≤ 60 kg, or serum creatinine ≥ 133 μmol/L (8).

Apixaban undergoes multiple pathways of elimination, which include: renal excretion (renal clearance accounts for 27% of its total clearance (9)), metabolism, as well as intestinal and biliary excretion (10, 11). Not surprisingly, factors that influence these elimination pathways have been shown to alter the pharmacokinetics of apixaban in subjects, and ultimately drug exposure (12, 13). For instance, individuals with impaired renal function exhibit lowered total clearance of apixaban and increased drug exposure such that subjects with mild, moderate, and severe renal impairment were associated with ~16%, ~29%, and ~44% higher area-underneath-the-curve (AUC) values based on regression analysis, respectively (12). Additional factors such as age and sex have shown to also influence the total clearance of apixaban, resulting in modest to low increases in drug exposure for subjects ≥65 years (32% higher AUC compared to those aged 18-40 years) and females (15% higher AUC compared to males) (13), with a difference in renal function between groups also thought to be contributing to these effects. More recently, functional genetic polymorphisms within genes that code for proteins involved in the transport (ATP binding cassette [ABC] G2 gene) and metabolism (cytochrome P450 [CYP]

3A5 gene) of apixaban were shown to be associated with drug exposure, and altered apixaban clearance (14, 15).

In terms of metabolism, apixaban is predominately hydroxylated by hepatic and intestinal cytochrome P450 3A enzymes (CYP3A4/5) (16, 17). Concomitant use of potent inhibitors and inducers of CYP3A4 such as ketoconazole and rifampin respectively, have been shown to alter apixaban AUC up to 50% (9, 18). In humans, 4β-hydroxycholesterol (4β-OHC) is an endogenous oxysterol that is created through the hydroxylation of cholesterol by CYP3A4 (19, 20). Studies have demonstrated that changes in plasma 4β-OHC concentrations correlate with changes in CYP3A4 activity driven by strong inducers and inhibitors of the enzyme (21, 22). Additional research has shown weak but significant correlation with other markers of CYP3A4 activity (midazolam (21, 23) and quinine/3-hydroxyquinine (24)). As such, 4β-OHC concentration may serve as an additional predictor of interindividual variability in apixaban exposure. However, to date, the relationship between apixaban blood concentrations and endogenous markers of CYP3A activity among patients taking apixaban, has not been determined. Accordingly, in this study we examined plasma 4β-OHC concentration − an emerging biomarker of CYP3A activity − as a predictor of apixaban plasma concentration within AF patients. Furthermore, we examined 4β-OHC concentration in combination with factors that are known to impact apixaban exposure such as renal function, age, sex, weight, and common genetic variation, in an attempt to better explain the interindividual variation in FXaI concentration observed within our apixaban-treated cohort.

4.2 Methods

4.2.1 Subjects and sample collection

Study protocol was approved by the Research Ethics Board of the University of Western Ontario (London, Canada), and written informed consent was obtained from study participants. Our study cohort consisted of apixaban-treated atrial fibrillation patients obtained from a hospital setting as previously described (25), with a blood sample collected at steady-state during regular apixaban dosing intervals.

4.2.2 Clinical and apixaban pharmacokinetic data

Patient blood was taken at steady-state with the following data collected at the time of draw: apixaban dosing regimen (2.5 or 5 mg twice daily), hours post-dose, age, sex, weight, serum creatinine (μmol/L), estimated creatinine clearance (mL/min, calculated using Cockcroft-Gault equation), concomitant medications. Serum creatinine data was missing for 5 individuals. Single time point apixaban plasma concentrations used in this study were obtained from our previously reported dataset (25).

4.2.3 Determination of 4β-hydroxycholesterol (4β-OHC) plasma concentration

Picolinic acid derivatization and ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) assay were used for measuring 4β-hydroxycholesterol (4β-OHC) as described by Honda et al. (26) with modifications. Both 4β-hydroxycholesterol and 4β-hydroxycholesterol-d7 (internal standard) were obtained from Avanti Polar Lipids (Alabaster, AL) and dissolved in methanol at 1 mg/mL. All other

chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Calibration standards of 4β-OHC (0, 5, 10, 20, 25, 30, 40, 50, 100, 200, 400, 800 ng/ml) were prepared in Krebs–Henseleit bicarbonate buffer. Standard or plasma sample aliquots (50 µL) were saponified using 500 µL of ethanolic KOH containing 4β-hydroxycholesterol-(d7) at 10 ng/mL on a shaker for 1 hour. Water (150 µL) was added to samples then extracted twice in 750 µL of hexane, with the organic layer collected and evaporated to dryness in a Savant SpeedVac concentrator (Thermo Scientific). Afterwards, 170 µL of the following derivatization reagents were added to each sample and incubated at 80˚C for 1 hour: 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), pyridine (1.5 µL), and trimethylamine (0.2 µL). Following the reaction, samples were once more extracted in 1 mL of hexane, and the organic layer was evaporated at 80˚C to dryness. Samples were reconstituted in 20 µL of NaCl dissolved in water (5 g/L) and 80 µL of acetonitrile, then 20 µL of the resulting mixture were injected into the UHPLC-MS/MS system. Chromatographic separation was performed using an Agilent 1290 Infinity ultra–high pressure liquid chromatography system (Agilent Technologies, Santa Clara, CA) and an Agilent Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 µm). Mobile phases used for gradient elution were 0.1% formic acid in water and 1:1 v/v acetonitrile:methanol. The mass analysis was performed on a TSQ Quantum triple-quadrupole mass spectrometer (Thermo Scientific), while the following mass transition were monitored in positive mode for quantification: 4β-hydroxycholesterol, 635.4 → 146.5 m/z; 4β-hydroxycholesterol-d7, 642.4 → 146.5 m/z. Interday variability on 3 separate days was 14.9%.

4.2.4 Single nucleotide polymorphism genotyping

Genomic DNA was extracted from participant blood sample using the Gentra Puregene Blood Kit (Qiagen, Alameda, CA). Genes encoding *ABCB1*, *ABCG2*, *CYP3A4* and *CYP3A5*, were assessed based on established associations to apixaban metabolism and disposition (16, 17, 27), as well as 4β-OHC metabolism (CYP3A4 and CYP3A5 only) (24). Accordingly, TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA) were used to genotype patients for the following-reduced functional polymorphisms: *CYP3A4* C>T Intron 6 (*22, rs35599367) (28), *CYP3A5* g.6986A>G (*3, rs776746) (29), ABCB1 c.3435C>T (rs1045642), and *ABCG2* c.421C>A (rs2231142). Hardy-Weinberg equilibrium was tested using χ^2 method in GraphPad Prism 6 (La Jolla, USA).

4.2.5 Data analysis

Nonparametic Spearman's correlation tests were performed to assess apixaban association with covariates 4β-OHC plasma concentrations, age, weight, estimated creatinine clearance (as shown in **Figure 4-1** and **Figure 4-2**). Differences in 4β-OHC with respect to moderate CYP3A4 inhibitor use were assessed by Kruskal-Wallis multiple comparisons. Significance was defined as two-tailed $p \leq 0.05$.

Log (base 10)-transformed apixaban plasma concentration was used as the dependent variable in our multivariable linear regression analysis. We included the following independent variables: age, sex, weight, serum creatinine (μmol/L), 4β-hydroxycholesterol (ng/ml), concomitant P-gp and CYP3A4 inhibitor use, transporter or enzyme polymorphism, apixaban dosing regimen (2.5 or 5 mg twice daily), and hours post-dose. Rows containing missing data were removed from the dataset, as were those patients who

were prescribed verapamil since there were too few patients to properly estimate the effect of this drug on apixaban concentration. This resulted in a dataset of 119 patients. We tested different genetic models for each genetic polymorphism – dominant, co-dominant, recessive, and additive models – and the model that best described the fit of apixaban plasma concentration was selected. With the exception of the *ABCB1* c.3435C>T polymorphism, SNP genotypes were treated as binary variables (dominant model) with those that were homozygous wildtype coded as 0, and those that were heterozygous or homozygous variant coded as 1. *ABCB1* c.3435C>T genotype was included as an additive model with those that were homozygous wild type, heterozygous, and homozygous variant coded as 0, 1, and 2, respectively. P-gp and CYP3A4 inhibitors, amiodarone and diltiazem were tested as separate binary variables, coded as 0 for non-users and 1 for users. Given that amiodarone and diltiazem use may have an effect on 4β-hydroxycholesterol concentration these variables were analyzed in separate models. We first regressed log (base 10) concentration onto dose, hours post-dose, age, sex, weight, serum creatinine, 4β-hydroxycholesterol, and transporter or enzyme polymorphism. We repeated this with the same independent variables substituting 4β-hydroxycholesterol for amiodarone and diltiazem use. Two outliers were identified when examining the models' residuals, the models' R^2 value improved after removing these outliers from the analysis.

4.3 Results

4.3.1 Study population characteristics and allele frequencies

We analyzed 4β-OHC plasma concentration data in 136 apixaban-treated AF patients with previously measured apixaban plasma concentration data (25). See **Table 4-1** for summary of patient characteristics. Thirty-one percent of subjects were also on a P-gp and CYP3A4 moderate inhibitor. Genotype and allele frequencies for the investigated *ABCB1*, *ABCG2*, *CYP3A4*, *CYP3A5*, polymorphisms are listed in **Table 4-2** with all genotype frequencies within Hardy-Weinberg equilibrium.

Table 4-1. Study population characteristics

Characteristic	
Subjects, n	136
Age, median (IQR)	75 (65-82)
Sex, Female (%)	46.3
Weight, kg, median (IQR)	82.7 (69.6-97.3)
BMI, kg/m2, median (IQR)	29.4 (24.6-34.0)
Apixaban Dose	
2.5 mg twice daily, (%)	53 (39.3)
5 mg twice daily, (%)	83 (61.5)
Hours post dose, mean (min-max)	6.4 (3-11.9)
Apixaban concentration, ng/ml, median (IQR)	187.6 (139.8-248.4)
Concomitant P-gp and CYP3A4 Inhibitor use, (%)	30.8
Amiodarone, (%)	13.4
Diltiazem and Verapamil, (%)	17.4
4β-hydroxycholesterol, ng/ml, median (IQR)	44.4 (28.8-71.1)
Serum Creatinine, μmol/L, median (IQR)	91 (72-111)
Creatinine Clearance, mL/min, median (IQR) ‡	62.5 (47.7-93.6)

Data represented as: percent of cohort (%), median (IQR, 25th and 75th percentile), mean (±SD). Abbreviations: BMI, body mass index; Lean body weight, LBW; IQR, interquartile range; SD, standard deviation. (‡) Creatinine clearance calculated using Cockcroft-Gault's formula for patients with available serum creatinine.

Table 4-2. Allele frequencies for the *ABCB1*, *ABCG2*, *CYP3A4*, and *CYP3A5* polymorphisms

Genotype	No. of patients	Genotype Frequency	Allele frequency
ABCB1 c.3435C>T			54.7%
C/C	25	0.197	
C/T	65	0.512	
T/T	37	0.291	
ABCG2 c.421C>A			12.8%
C/C	98	0.76	
C/A	29	0.225	
A/A	2	0.016	
CYP3A4 *22			7.5%
C/C	109	0.858	
C/T	17	0.134	
T/T	1	0.008	
CYP3A5 *3			94.6%
A/A	0	0	
A/G	14	0.109	
G/G	115	0.891	

4.3.2 4β-hydroxycholesterol plasma concentration

We observed a 38.3-fold variation (minimum to maximum) in the measured plasma 4β-OHC concentrations of our AF patients, with median of 44.4 ng/ml (13.5 - 131.6, 5-95th percentile). When comparing the 4β-OHC in relation to apixaban we found there was a statistically significant (p = 0.016, r = -0.2063) weak inverse correlation between their measured plasma concentrations (**Figure 4-1A**). Apixaban concentrations did not significantly correlate (p = 0.2047) with 4β-OHC concentrations when they normalized to total cholesterol (**Figure 4-1B**).

We also compared 4β-OHC concentrations of subjects that were taking moderate CYP3A4 and P-gp inhibitors to non-users (**Figure 4-1C**). Those taking diltiazem had 40% lower 4β-OHC concentrations compared to non-users (p < 0.0045), however, no significant difference was observed for amiodarone users. We did not observe a statistically significant difference in 4β-hydroxycholesterol plasma concentrations between wildtype and carriers of *CYP3A4**22 and *CYP3A5**3 alleles (data not shown).

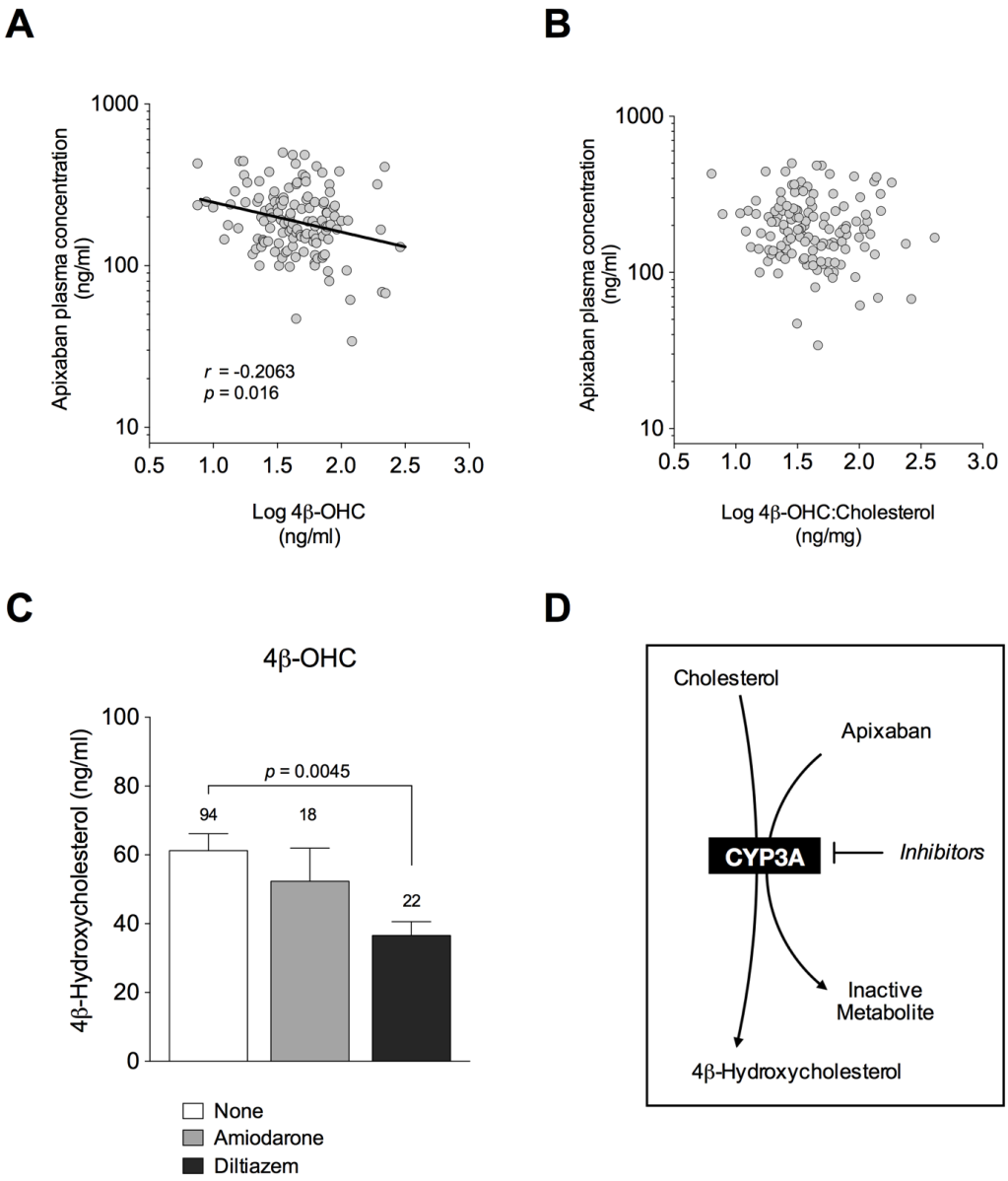


Figure 4-1. Relationship between apixaban and 4β-hydroxycholesterol plasma concentrations.

Association between apixaban (single time point) plasma concentrations with (A) 4β-OHC and (B) cholesterol normalized ratio. (C) Mean (±SD) 4β-OHC plasma concentrations in subjects taking concomittant CYP3A4 inhibitors compared to non-users. (D) Proposed mechanism for a assiocation between 4β-OHC, apixaban, and exogenous CYP3A4 inhibitors. Abbreviations: 4β-OHC, 4β-hydroxycholesterol; r, Spearman correlation coefficient; p, p-value.

4.3.3 Apixaban plasma concentration

We assessed the correlation between apixaban plasma concentration and the clinical factors used for dose selection. After normalizing for dosage, apixaban plasma concentrations positively correlated with increasing age ($r = 0.3455$, $p < 0.0001$) and increasing serum creatinine ($r = 0.3416$, $p < 0.0001$) (**Figure 4-2A-B**). While patients that weighed less appeared to have higher drug concentrations, this was not statistically significant (**Figure 4-2C**). Furthermore, plasma concentrations were found to correlate more with Cockcroft-Gault's estimated creatinine clearance ($r = -0.4984$, $p < 0.0001$; **Figure 4-3**), a formula used to estimate glomerular filtration rate using age, serum creatinine, weight, and sex.

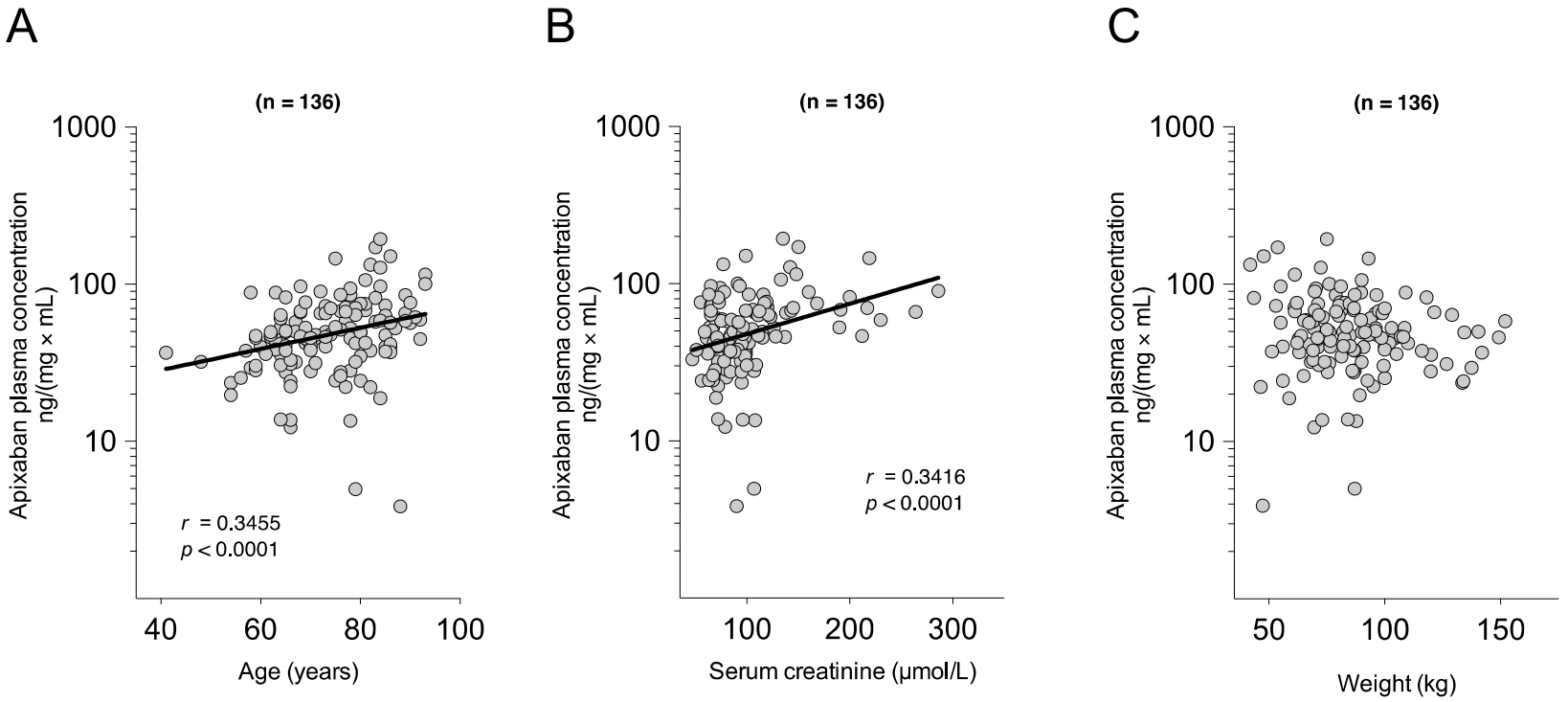
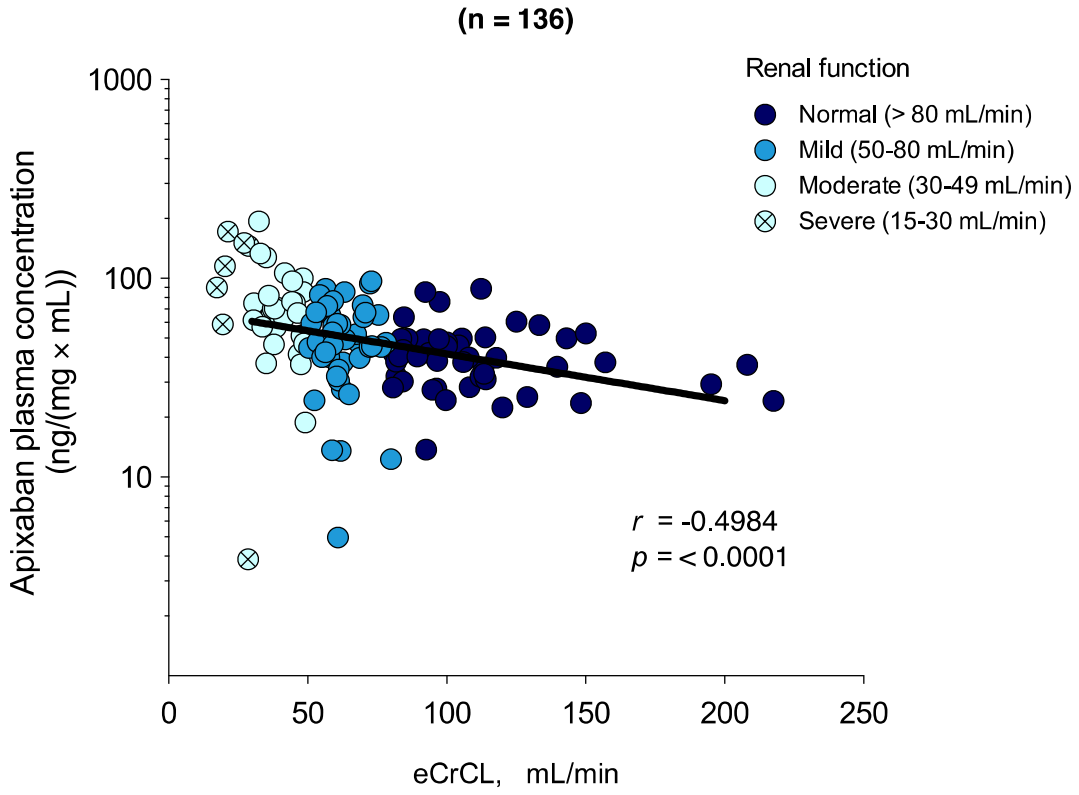


Figure 4-2. Relationship between and apixaban and clinical factors used for dose selection.

Dose-normalized apixaban (single time-point) plasma concentrations versus clinical factors used for apixaban dosing: (A) age, (B) serum creatinine, (C) serum creatinine. Abbreviations: r, Spearman correlation coefficient; *p*, *p*-value.



$$\text{eCrCl}_{\text{Cockcroft-Gault formaula}} = \frac{(140 - \text{age}) \times \text{weight}}{0.814 \times \text{Serum creatinine (µmol/L)}} \times \underset{\text{(if female)}}{0.85}$$

Figure 4-3. Relationship between apixaban and estimated creatinine clearance.

Dose-normalized apixaban (single time-point) plasma concentrations versus estimated creatinine clearance. eCrCl was calculated using Cockcroft-Gault's formula. Abbreviations: eCrCl, estimated creatinine clearance; r, Spearman correlation coefficient; p, p-value.

4.3.4 Multivariable linear regression analysis

Multivariable linear regression analysis was carried out to assess the relative contribution of 4β-OHC as determinant of apixaban plasma concentration while controlling for clinical variables (age, sex, weight, serum creatinine, apixaban dose, hours post dose) as well as genetic polymorphisms in *CYP3A4*, *CYP3A5*, *ABCB1*, and *ABCG2*. Our regression analysis indicated higher 4β-OHC concentrations were associated with lower apixaban concentrations, even after controlling for these covariates (p = 0.001; **Table 4-3**). The adjusted r^2 value (percent explained) of the model was 0.279. Higher apixaban concentrations were associated with increased age (p = <0.001), female sex (p = 0.014), and elevated serum creatinine (p = <0.001). Hours post dose, weight, diltiazem use, as well as CYP3A4*22, CYP3A5*3, ABCB1 c.3435C>T, or ABCG2 c.421C>A carrier status were not significant predictors (p > 0.05) of apixaban plasma concentration in our cohort. Ultimately, the addition of 4β-OHC as a variable increased the variation explained by 7.3% (27.9% versus 20.6% total variation explained) compared to a model with only clinical and genetic variables (**Figure 4-4**).

The effect of amiodarone and diltiazem use were assessed in a separate regression model excluding 4β-OHC (Figure 4-4). The results of this analysis indicated amiodarone use (p = 0.014) and by not diltiazem use to be associated with higher apixaban plasma concentration (**Appendix C**). This model explained 23.5% of interpatient variation in apixaban plasma concentration.

Table 4-3. Multiple linear regression coefficients for apixaban plasma concentration in atrial fibrillation patients (n = 119).

Predictor variable	**B**	**95% CI**	***P*-value**
Dose	0.111	0.076, 0.146	<0.001
Hours post dose	-0.013	-0.031, 0.005	0.152
Age, years	0.007	0.003, 0.011	0.001
Sex, Female	0.092	0.019, 0.166	0.014
Weight, kg	-0.001	-0.003, 0.001	0.461
Serum creatinine, µmol/L	0.002	0.001, 0.003	<0.001
ABCB1 c.3435C>T , per allele	0.002	-0.046, 0.051	0.925
ABCG2 c.421C>A, 1 or 2 allele	0.068	-0.009, 0.145	0.083
*CYP3A4**22, 1 or 2 allele	0.010	-0.084, 0.103	0.837
*CYP3A5**3, 1 or 2 allele	0.016	-0.092, 0.124	0.771
4β-Hydroxycholesterol, ng/ml	-0.001	-0.002, -0.001	0.001
Adjusted *R* squared for model, 0.279			

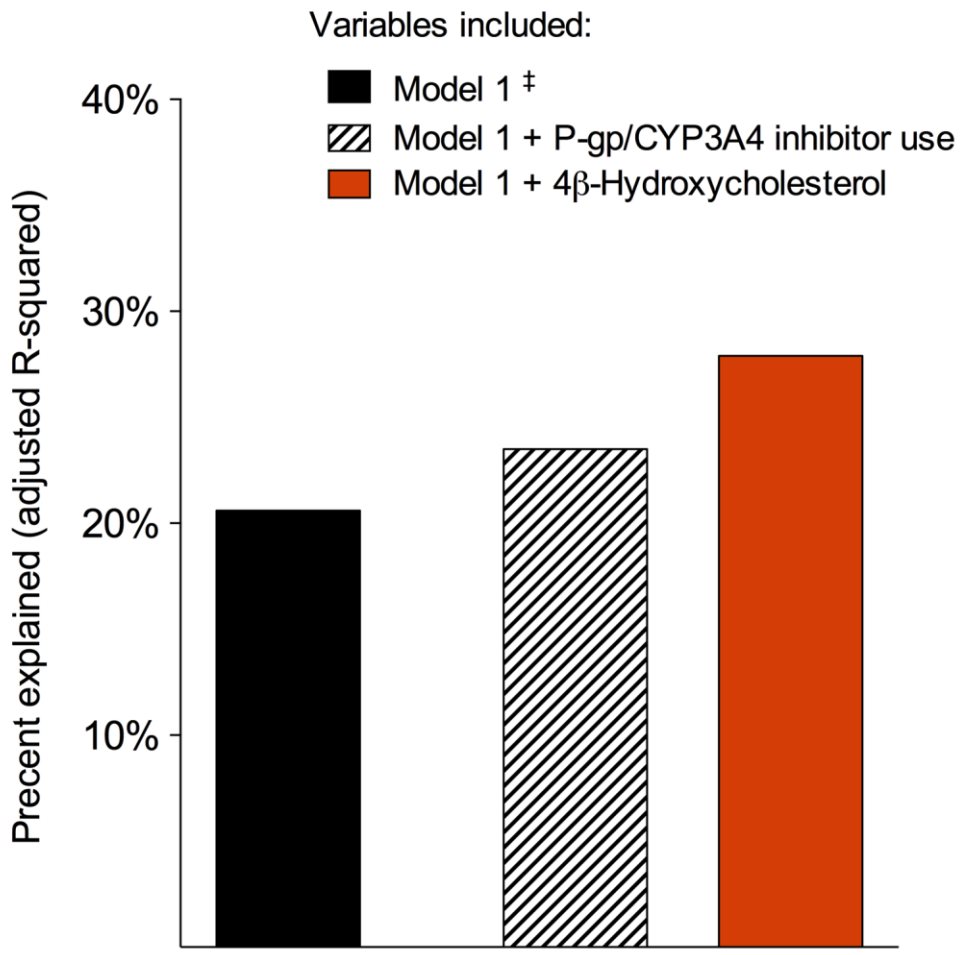


Figure 4-4. Interpatient variation in apixaban plasma concentration explained by clinical variables, genetic polymorphisms, and 4β-hydroxycholesterol

Nested model comparing the percent of interpatient variation in apixaban plasma concentration explained with or without including 4β-Hydroxycholesterol. (‡) Model 1 including independent variables: dose, hours post dose, age, sex, weight, serum creatinine, as well as *CYP3A4*, *CYP3A5*, *ABCB1*, and *ABCG2* genotype.

4.4 Discussion

Apixaban is primarily oxidized by CYP3A enzymes (14, 15). Accordingly we examined plasma 4β-OHC concentration − an emerging biomarker of CYP3A activity − as a predictor of interindividual variability in apixaban plasma concentration among AF patients. We found a weak, but statistically significant correlation between plasma concentrations of apixaban and 4β-OHC, with higher 4β-OHC concentrations (higher CYP3A4 activity) associated with lower apixaban concentrations (**Figure 4-1, Table 4-3**). Even though the majority of the interindividual variation in apixaban concentration remained unexplained (~72%), including 4β-OHC along with clinical variables in our multivariable linear regression analysis improved the variation explained by 7% (28% versus 21%) (**Figure 4-4**), when compared to a model with clinical variables alone. Interestingly, 4β-OHC:cholesterol (cholesterol normalized ratio) did not show any statistically significant correlation with apixaban concentration.

4β-hydroxycholesterol (4β-OHC) is one of the dominate oxysterols present in the human circulation at concentrations 10^3-10^4 times lower than total cholesterol with a reported half-life ≥ 60 hours (30, 31). The role of CYP3A4 in the hydroxylation of cholesterol to 4β-OHC was demonstrated in the early 2000s by Bodin and colleagues through in vitro incubation of cholesterol with recombinant CYP enzymes (CYP3A4, CYP1A2, CYP2C9, CYP2B6) (32). Among the enzymes tested, 4β-OHC formation was found only with CYP3A4. This effort came after the discovery that patients taking carbamazepine, phenytoin, and phenobarbital (strong inducers of CYP3A) experienced an ≥ 20-fold increase in plasma 4β-OHC concentration (32). Since then, many studies have shown that changes in 4β-OHC correlate well with changes in CYP3A activity that are

driven by strong CYP3A inducers (19, 23, 33-35) and inhibitors (35-37), with 4β-OHC increasing and decreasing from baseline, respectively. In our study, subjects that were taking the moderate inhibitor, diltiazem (n = 22), had significantly lower 4β-OHC concentration (40% lower compared to non-users, n = 94). However, no difference was seen in amiodarone users, which is consistent with diltiazem having greater evidence for a CYP3A4 inhibitory effect compared to amiodarone (38, 39).

The evidence surrounding 4β-OHC supports its utility for assessing changes in the CYP3A activity that are driven by strong CYP3A inhibitors and inducers. On the other hand, its usefulness as a marker of basal CYP3A activity is yet to be fully established (40). Current evidence shows a weak correlation between 4β-OHC concentrations and previously recognized markers of CYP3A activity (midazolam (21, 23) and quinine/3-hydroxyquinine (24)), but its association with other exogenous CYP3A substrates have produced mixed results. In an study looking at anticancer drug, docetaxel (largely metabolized by CYP3A4), the authors found 4β-OHC to have limited predictive value towards docetaxel clearance, with a correlation between 4β-OHC concentration and clearance seen only in males ($r = .35$, $p = 0.01$) (41). Our group has previously reported a relationship between 4β-OHC and plasma concentrations of the lipid-lowering drug, atorvastatin, also known to be metabolized by CYP3A4 (42). Taken together, the degree of association between 4β-OHC and exogenous substrates is more than likely complicated by factors other than CYP3A that include, but are not limited to: differences solubility and permeability, intestinal *versus* hepatic metabolism, and substrate specificity to intestinal, hepatic, or renal transporters.

Although CYP3A-mediated metabolism is an elimination pathway for apixaban, it is not the only way that this drug is cleared from the body. In a mass-balance study with healthy subjects, only ~30% of the recovered apixaban dose (after oral administration) was in metabolite form (10). This may reflect why 4β-OHC was observed to have a marginal effect in explaining the variation of apixaban plasma concentration among our AF patients.

Kidney function and age were the strongest predictors of variation in apixaban concentration among our AF cohort. The correlation of apixaban plasma concentration with serum creatinine (**Figure 4-2B, Table 4-3**) and estimated creatinine clearance (**Figure 4-3**) that we observed is consistent with phase I study results, which showed worsening renal impairment correlated with reduced total clearance and increased AUC. Furthermore, our observation of higher apixaban concentration being associated with increased age (**Figure 4-2A, Table 4-3**) and female sex (**Table 4-3**), were also consistent with previous studies involving healthy (13), AF (14), and VTE (43) subjects. Although extremes in body weight (≤ 50 kg or ≥120 kg) have previously been shown to influence apixaban exposure (44), weight was not a predictor of variation in apixaban concentration among our patients. This likely due to the fact that only 12% of our patients were ≤ 50 kg or ≥120 kg. In addition to the CYP3A enzymes, apixaban is a known substrate of ATP binding cassette (ABC) efflux transporters, P-glycoprotein (P-gp, ABCB1) and Breast Cancer Resistance Protein (BCRP, ABCG2) (14, 15). While we did not observe an association between carrier status of common functional genetic polymorphisms and variability in apixaban plasma concentration, recent work in a Japanese AF population report the ABCG2 421A/A genotype to be associated with higher trough apixaban plasma concentrations and reduced clearance, whilst the CYP3A5*1/*1 genotype was seen to be associated with lower trough

concentration and higher clearance (14, 15). This discrepancy is likely due to the differences in the allele frequencies that are exist between the different ethnic populations (Ueshima et al. allele frequencies were 33% and 78% for *ABCG2* 421C>A and CYP3A5*3 respectively). The effect of genetic variation may have been observed had there been more variant carriers in our study.

In summary, our results are consistent with what is known about the CYP3A4-mediated metabolism of apixaban and 4β-OHC formation. Even though the clinical factors used for dose selection, such as age and serum creatinine, accounted for the majority of the explainable variation in apixaban plasma concentration, our findings suggest that CYP3A4 activity can be used as an additional predictor of interindividual variability of apixaban exposure. Indeed, further investigations correlating 4β-OHC as well as other markers of CYP3A4 activity with parameters of apixaban exposure (i.e., AUC, trough plasma concentrations) and clearance are warranted to confirm the degree to which CYP3A4 activity influences apixaban exposure.

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5 Identification of rare or patient-specific single nucleotide variants as potential determinants of apixaban disposition using a custom targeted next-generation exome sequencing approach

Gulilat M, Lamb T, Teft WA, Robinson JF, Tirona RG, Hegele RA, Kim RB, Schwarz UI. Targeted next generation sequencing as a tool for precision medicine. Manuscript in preparation, 2018.

5.1 Introduction

In Chapter Four, we noted that known common genetic variation in metabolizing enzymes and efflux transporters (*CYP3A4*22*, *CYP3A5**3, *ABCB1* c.3435C>T, or *ABCG2* c.421C>A) were not significant predictors of the interpatient variation in apixaban concentration observed in our AF cohort. Furthermore, a large proportion (~72%) of this variation remained unaccounted for within our regression analysis. It is our hypothesis that rare or patient-specific single nucleotide variations within certain individuals are likely contributing to this variability.

While previous pharmacogenetic research has focused on evaluating common functional variation, more recent large-scale whole genome or exome sequencing studies have revealed that humans harbor a large number of rare, potentially deleterious variants in many of these pharmacogenes (1, 2). Specifically, the analysis of sequencing data for 146 pharmacogenes combining about 7500 individuals of the Exome Sequencing Project (ESP) (3) and the 1000 Genomes Project (1000G) (4) indicated that more than 90% of all recorded single nucleotide variants (SNVs) were rare with a minor allele frequency (MAF) below 1%, and that 30-40% of the predicted functional variability was associated with these rare variants (1). Further studies also support that rare SNVs in drug processing or drug target genes significantly contribute to interpatient differences in drug disposition and response beyond established common genetic predictors as shown for *CYP2C9* and warfarin dose requirement (5, 6) and *SLCO1B1* and methotrexate clearance and toxicity (7).

Targeted next-generation sequencing (NGS) represents a new time- and cost-effective technology for detecting common and rare genetic variation, allowing for DNA

enrichment of entire exomes or coding exons of select genes coupled with massive parallel sequencing of the enriched product. Through the application of this method, particularly, in those apixaban-treated AF subjects who demonstrated unexpectedly high drug plasma concentrations, we would be able to better identify those individuals with rare SNVs that result in aberrant function or expression of their CYP enzyme or transporter, consequently impacting their apixaban exposure.

Therefore, we created a NGS-based exome capture panel (PGxSeq) capable of detecting common and clinically established, as well as rare and potentially functional genetic variation within 100 pharmacogenes, including seven genes (*ABCB1, ABCG2, CYP3A4, CYP3A5, POR, NR1H4, NR1I2*) that are relevant to apixaban disposition. Our objectives were to: 1) evaluate the sequencing performance as well as the accuracy of variant discovery within a validation cohort (n = 48); 2) evaluate the identified variation in all 100 pharmacogenes with respect to population allele frequencies and predicted functional effects for a larger cohort (n = 245); 3) apply this method for SNV discovery in a small exploratory cohort of apixaban-treated AF patients who exhibited FXaI plasma concentrations exceeding the maximum concentration observed during clinical trials while being dosed according to product monograph (test group n = 6, control n = 6).

5.2 Methods

A flow diagram of the sample and data processing can be found in **Figure 5-1**. A detailed methods description can be found in **Appendix D**.

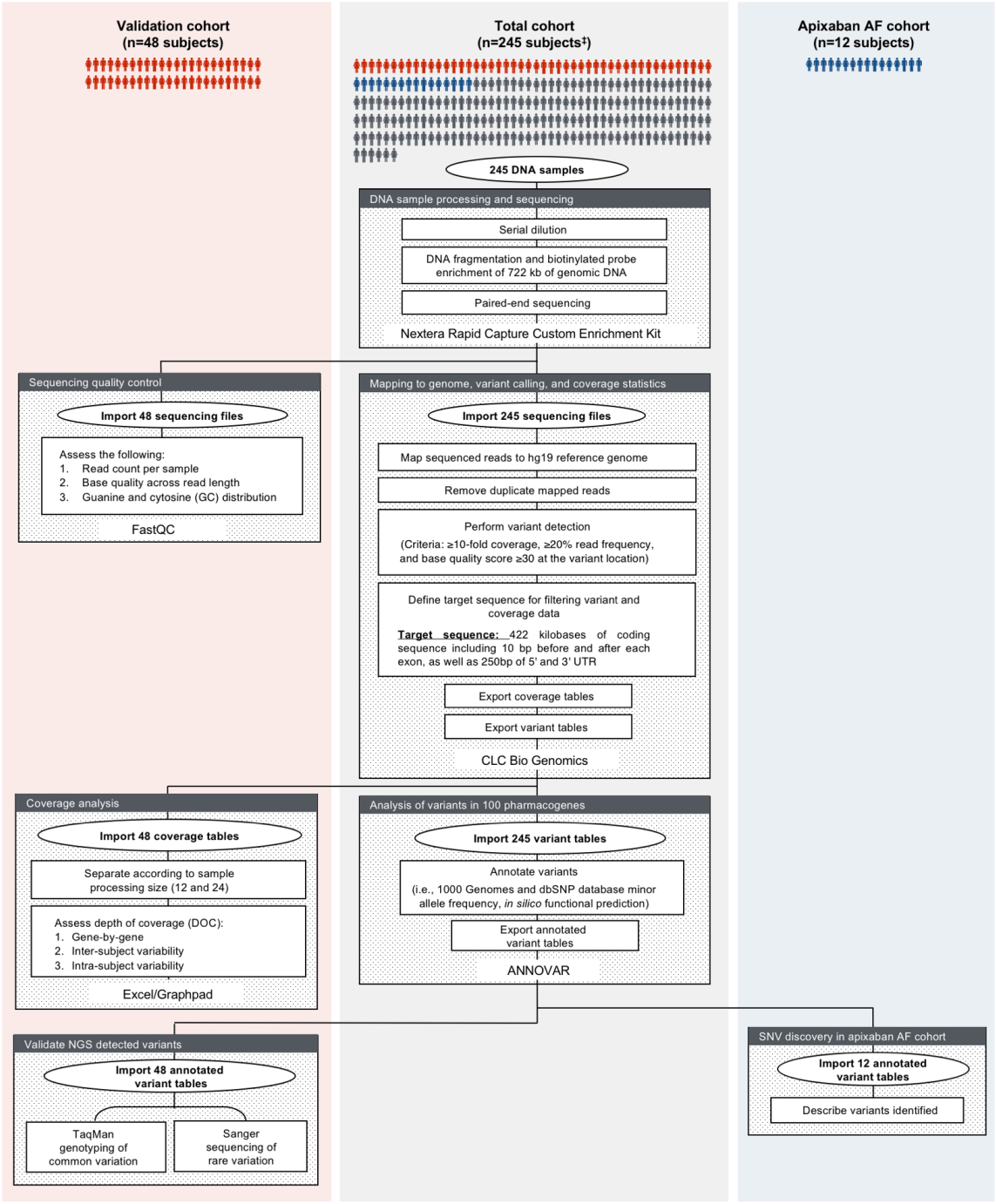

Figure 5-1. Sample and data processing workflow. ‡
One subject was sequenced twice to obtain higher sample depth-of-coverage.

5.2.1 Study population

NGS was carried out in a total of 245 Caucasian subjects, for which genomic DNA was obtained from a venous blood sample following written informed consent. Sequencing performance and accuracy of variant detection was evaluated in the first 48 subjects − this was our validation cohort. Following this, all 245 subjects were used to evaluate genetic variation in terms of population allele frequencies and predicted functional effect. Then we carried out candidate gene SNV discovery in a subgroup of 12 apixaban-treated AF patients (described below) with a known apixaban drug concentration phenotype. This study was approved by the Research Ethics Board of Western University, London, Canada.

5.2.2 Apixaban cohort

Twelve individuals from this previous study were included as part of the n = 245 participants sequenced in this study, and consisted of two different apixaban plasma concentration phenotypes: high concentration (test group, n = 6) and normal concentration (control group, n = 6). Specifically, apixaban phenotype was determined based on whether the patient's measured apixaban plasma concentration fell within the range of 41 - 321 ng/mL, representing the lower limit for C_{min} (5^{th} percentile) to the upper limit for C_{max} (95^{th} percentile), respectively, for an individual on apixaban 5 mg twice daily at steady state as previously described (refer to Chapter Four). Concentrations above this range were considered high, while those within were considered normal. The inclusion criteria for both groups was that patients were dosed according to product monograph, and did not meet any of the clinical factors used for dose reduction that are known clinical predictors of

higher apixaban exposure (age ≥ 80 years, body weight ≤ 60 kg, or serum creatinine ≥ 133 µmol/L)(8).

Our goal was to discover rare or patient specific hypothesis generating single nucleotide variations among candidate genes involved in the metabolism or transport of apixaban that potentially correlate with apixaban concentration phenotype. The candidate genes analyzed were selected based on the following evidence: 1) the gene product interacts directly with apixaban as a transporter (*ABCB1, ABCG2*) or metabolizing enzyme (*CYP3A4, CYP3A5)*; or 2) the gene product is involved in regulating the expression (*NR1H4*, *NR1I2*), or is required for the function (*POR*) of an enzyme or transporter that interacts directly with apixaban.

5.2.3 Gene selection, capture probe design and enrichment method

DNA enrichment was carried out using the Nextera Rapid Capture Custom Enrichment Kit (Illumina, San Diego, CA) comprising of 100 genes including major cytochrome P450 (CYP) enzymes, phase II conjugation enzymes, drug transporters of the solute carrier (SLC) and ATP binding cassette (ABC) families as well as regulatory proteins. In addition, regions encompassing 14 known functional promoter or intronic SNVs were included. Capture probes were custom-designed using the Illumina Design Studio (Illumina, San Diego, CA) comprising of 722 kilobases (kb) with 4% overlap. DNA library preparation and target-capture sequencing was conducted at the London Regional Genomics Center, London, Ontario, as previously described (9).

5.2.4 Base calling, sequence alignment, coverage analysis and variant detection

Alignment of sequencing reads and variant calling were performed using the CLC Bio Genomics Workbench 7.0 (CLC Bio, Aarhus, Denmark) through a custom-automated workflow. Coverage analysis in the validation cohort and variant detection in all 245 subjects was restricted to coding regions plus 10-bp before and after each exon and 250-bp of 5' and 3' UTR, and targeted promoter or intronic SNVs.

5.2.5 Variant annotation and *in silico* prediction

Functional annotation of SNVs was carried out using ANNOVAR (10) through *in silico* prediction algorithms (Combined Annotation Dependent Depletion [CADD] (11), Sorting Intolerant from Tolerant [SIFT] (12), and PolyPhen-2 (13)), and population frequency determined through linkage with genomic databases (RefSeq, dbSNP137, 1000G, Exome Aggregation Consortium [ExAC]; obtained July 8, 2015). Potentially functional SNVs (or deleterious) were considered with a score of >20 for CADD (scaled) (11), <0.05 for SIFT (12), or >0.85 for PolyPhen-2 (13). Variants were classified as non-synonymous, synonymous, insertion or deletion (in-del; frameshift, non-frameshift), splicing, stop gain or loss, or known functional intronic/ promoter variants as non-coding. Coding variants were further grouped by MAF reported in the ExAC database as common (MAF $\geq$ 5%), low frequency (1% $\leq$ MAF > 5%), or the combined category of rare (MAF < 1%) and novel (absent from ExAC and dbSNP137). *In silico* functional assessment was restricted to protein-coding and gain- or loss-of-a-stop codon SNVs.

5.2.6 Concordance assessment

In our validation cohort, orthogonal genotyping was performed using TaqMan allelic discrimination for 32 functionally validated or clinically actionable SNVs. Rare NGS variants were confirmed retrospectively by Sanger sequencing within 4 pharmacogenes. Sanger sequencing methods and TaqMan assay IDs are listed in **Table S2, Appendix D,** and **Table S3, Appendix D**, respectively.

5.2.7 Statistical analysis

Analyses were performed using R (14) and GraphPad Prism 6 (La Jolla, USA). Differences in the proportion of possibly deleterious variants grouped by frequency was assessed using Chi-squared test, and Pearson correlation coefficient used to determine the association between allele frequencies in our study compared to 1000 Genomes and ExAC database. Statistical significance was defined as $P<0.05$.

5.3 Results

5.3.1 Sequencing performance and accuracy of variant detection in the validation cohort

Performance parameters by sequencing run for the first 48 subjects are presented in **Table 5-1**. The total number of sequenced reads per run ranged from 20.0 to 27.6M reads, with 87.1 to 89.2% of reads passing Illumina control filters (Reads PF). The mean per base quality score (Phred quality score, Q) for Reads PF were ≥30, indicating a miscall error rate in base calling of less than 0.1% (or 99.9% sequencing accuracy) up until the 150 bp read position. A decline in base quality was observed beyond 150 bp likely due to a combination of short fragment length with the 2 x 300 bp chemistry used for DNA library preparations (**Figure S1, Appendix E**). Consequently, the threshold for variant detection was set to Q ≥ 30 to minimize false-positive variant calls. After de-clustering each run, sequencing data for each sample were assessed for read count, base quality, and guanine (G) and cysteine (C) distribution (**Figure 5-1**). Three samples had a mean GC content of up to 65%, exceeding the expected range of 45-50% (15) (**Figure S2, Appendix E**).

Table 5-1. Overview of sequencing performance parameters in our validation cohort separated by run cluster.

	Run 1	Run 2	Run 3
Raw sequencing parameters (n = 48)			
Subjects, n	12	12	24
Cluster density, (1000/mm2)	841	1149	1183
Total reads, (10^6)	20	27.1	27.6
Reads PF, (10^6)	17.8	23.9	24
Reads PF, (%)	89.2	88.2	87.1
Mapping parameters (422 kb)			
Subjects, n	12	12	24
Reads PF mapped to hg19 genome (10^6)			
per sample	1.2	1.6	1
DOC, mean (- duplicates removed)	187.3 [a]	297.7 [a]	127.9 [a]
DOC, median (- duplicates removed)	187 [a]	303 [a]	126 [a]
Bases with mean DOC <30x, (%)	2.4 [b]	1.8 [b]	3.3 [b]

Reads PF, reads passed filter; DOC, depth of coverage. [a] Calculated for samples across the 422 kb target sequence; [b] Calculated as percent of 422 kb target sequence. [‡] Excluding one sample with low read count (< 2500 reads).

We next assessed the depth-of-coverage (DOC), defined as the number of overlapping non-duplicate reads mapped to the reference genome, across the target sequence separated by sequencing cluster (n = 12 or 24). As expected, samples in the smaller cluster had a greater mean DOC per subject (range 183 – 298×) compared to those sequenced in the larger 24 DNA sample cluster (128×) (**Table 5-1**). Despite these differences, the percent bases with a mean DOC < 30× was comparable ranging from 1.8% - 3.3%. When evaluated on a gene-by-gene basis (**Figure 5-2**), 98 of the 100 genes on our panel had a mean DOC ≥ 80×. Among all genes, the glutathione S-transferase (GST) genes showed the lowest DOC per subject (min-max; 14.1 - 233× for *GSTM1*; 5.5 - 267× for *GSTT1*). Carboxylesterase 1 (*CES1)* had the highest proportion of targeted regions < 30×, largely due to lack of coverage for exons 12 to 14, followed by carbonyl reductase 3 (*CBR3*), and *CYP1A2* (**Figure S3, Appendix E**). The remaining genes of Phase I and II enzymes, ABC and SLC transporters, nuclear receptors, and drug targets had ≥ 95% of their target region at DOC > 30×. Intra-subject variability in DOC was greatest in the 3 DNA samples with high GC content (**Figure S4, Appendix E**).

Utilizing TaqMan assays for 32 validated or clinically actionable SNVs in our validation cohort (**Table 5-2.**), we observed 100% concordant between TaqMan derived *versus* NGS derived genotypes, confirming heterozygous and homozygous carrier status. Using Saner sequencing we were able to confirm all five rare coding variants that were identified by NGS (**Table 5-2.**).

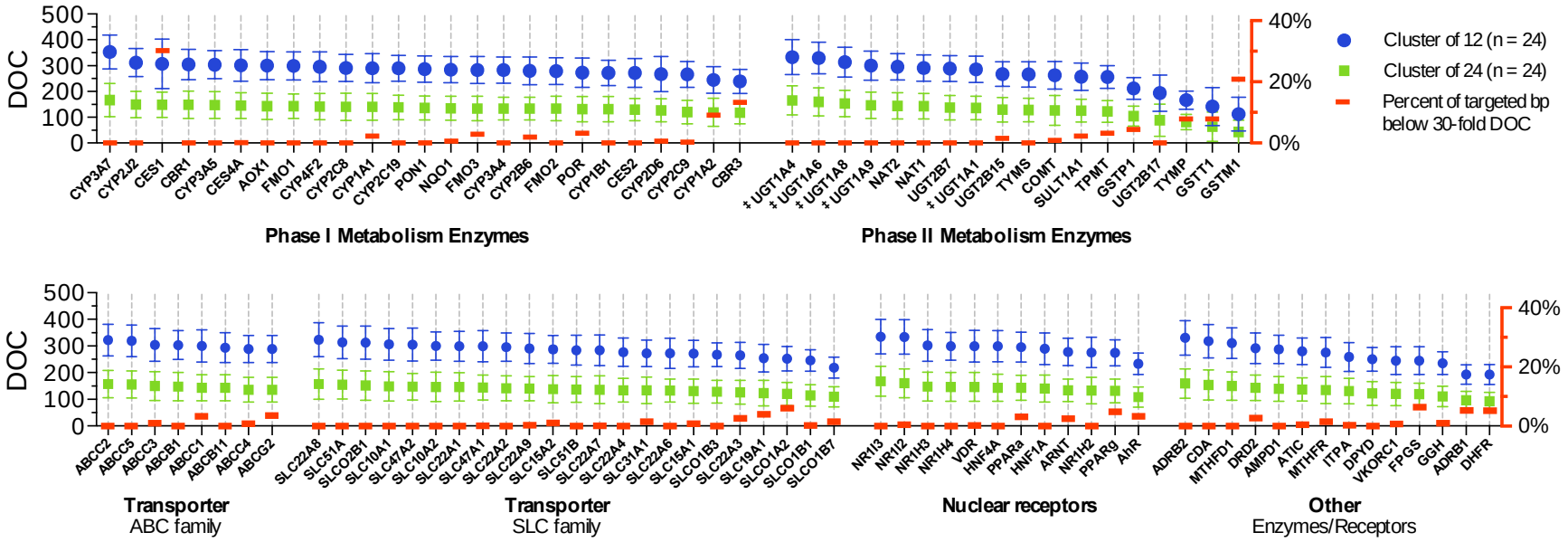

Figure 5-2. Depth of coverage per gene.

Depth-of-coverage (DOC) by gene separated by size of sequence cluster (n=12 or 24). ‡ For *UGT1A4*, *UGT1A6*, *UGT1A8*, and *UGT1A9*, the first exon was included to calculate DOC while shared exons were assessed only once with *UGT1A1*. Data are shown as mean ±SD

Table 5-2. Concordance rate (%) of genotypes between NGS data and TaqMan or Sanger validated genotypes in the validation cohort (n = 48).

Gene	Allele	Polymorphism	Effect/ Location	dbSNP137	Study (n=245) AF	1000G EUR AF	EXaC AF	Patients tested HET	Patients tested HOM	Concordance (%)[a]
A. Concordance of common and/or clinically relevant SNPs with TaqMan genotyping										
ABCB1		c.3435 C>T	p.I1145I	rs1045642	0.47	0.47	0.50	11	3	100
ABCC2		c.1249 G>A	p.V417I	rs2273697	0.18	0.20	0.20	5	2	100
ABCG2		c.421 C>A	p.Q141K	rs2231142	0.11	0.10	0.12	10	3	100
ABCG2		c.34 G>A	p.V12M	rs2231137	0.04	0.06	0.11	2	-	100
CYP2B6		c.1459 C>T	p.R487C	rs3211371	0.12	0.10	0.09	8	1	100
CYP2B6	***9**	c.516 G>T	p.Q172H	rs3745274	0.22	0.23	0.27	5	2	100
CYP2C19	***17**	c.-806 C>T	promoter	rs12248560	0.22	0.23	NA	8	3	100
CYP2C19	***2**	c.681 G>A	p.P227P	rs4244285	0.14	0.15	0.19	4	1	100
CYP2C9	***2**	c.430 C>T	p.R144C	rs1799853	0.13	0.12	0.09	7	2	100
CYP2C9	***3**	c.1075A>C	p.I359L	rs1057910	0.06	0.06	0.06	5	-	100
CYP2D6	***3A**	g.2549 A>del	p.R208fs	rs35742686	0.03	0.02	0.01	2	-	100

CYP2D6	***4***	g.1846 G>A	splice	rs3892097	0.19	0.19	0.17	16	4	100
CYP2D6	***9***	c.688_690AAG>del	p.K281del	rs5030656	0.02	0.02	0.02	1	-	100
CYP2D6	***10***	g.100 C>T	p.P34P	rs1065852	0.22	0.20	0.25	18	4	100
CYP2D6	***41***	g.2988 G>A	intronic	rs28371725	0.12	0.09	0.08	10	1	100
CYP3A4	***22***	c.522-191 C>T	intronic	rs35599367	0.05	0.05	NA	7	-	100
CYP3A5	***3***	g.6986 A>G	splice	rs776746	0.06	0.95	NA	6	-	100
CYP4F2	***3***	c.1297 C>T	p.V433M	rs2108622	0.30	0.27	0.27	6	3	100
DPYD		c.2846 A>T	p.D949V	rs67376798	0.02	0.0022	0.0026	1	-	100
NR1H4/FXR		c.-1 G>T	5' UTR	rs56163822	0.03	0.02	0.08	4	-	100
NR1H3/LXRa		c.297 C>T	p.S547S	rs2279238	0.16	0.16	0.25	6	2	100
POR		c.1508 C>T	p.A503V	rs1057868	0.26	0.30	0.36	5	2	100
NR1I2/PXR		g.39823 C>T	intronic	rs2276707	0.20	0.17	0.24	9	-	100
SLCO1B1	***1b***	c.388 A>G	p.N130D	rs2306283	0.39	0.40	0.48	9	4	100
SLCO1B1	***5***	c.521 T>C	p.V174A	rs4149056	0.17	0.17	0.13	9	2	100
SLCO1B3		c.699 G>A	p.M233I	rs7311358	0.80	0.85	0.80	5	16	100

SLCO2B1		c.935 G>A	p.R290Q	rs12422149	0.07	0.10	0.17	7	-	100
TPMT	***3A***	c.460 G>A	p.A154T	rs1800460	0.04	0.03	0.03	4	-	100
TPMT	***3B***	c.719 A>G	p.Y240C	rs1142345	0.04	0.03	0.04	4	-	100
VDR		g.1270 G>A	intergenic	rs11568820	0.17	0.23	NA	3	1	100
VDR		g.47906043 T>C	intergenic	rs4516035	0.43	0.43	NA	8	7	100
VKORC1		c.-1639 G>A	intergenic	rs9923231	0.39	0.40	NA	5	6	100
B. Concordance of rare variation in select genes by Sanger sequencing										
ABCB1		c.3320 A>C	p.Q1107P	rs55852620	0.012	1.0E-02	6.1E-03	1	-	100
CYP2D6		c.A818C	p.H273P	rs5030867	0.002	NA	0.0013	1	-	100
SLCO1B1		c.850 A>C	p.N284H	novel	0.002	NA	4.1E-05	1	-	100
SLCO1B1		c.1159 G>A	p.A387T	novel	0.002	NA	1.7E-05	1	-	100
SLCO1B3		c.287 T>G	p.L96X	novel	0.002	NA	NA	1	-	100

([a]) Percentage of total tested DNA samples with NGS-determined genotypes concordant with TaqMan or Sanger derived genotypes. Abbreviations: AF, allele frequency; dbSNP 137, Single Nucleotide Polymorphism database build 137; ExAC, Exome Aggregation Consortium version 0.3; HET, heterozygous genotype; HOM, homozygous genotype; 1000G EUR, 1000 Genomes Project European dataset; NA, not found in dbSNP 137, 1000G EUR, or ExAC database, respectively

5.3.2 Analysis of variants in pharmacogenes

Genetic variation was assessed in 245 Caucasians (**Figure 5-1, 5-3**), and the SNVs identified presented in **Figure 5-3** according to functional effect, number of variants per gene, or reported MAF in ExAC (if exonic). A total of 1089 unique SNVs were identified, consisting of 605 non-synonymous (55.6%), 409 synonymous (37.6%), 13 splice-site (1.2%), 13 stop gain or loss (1.2%), and 37 insertion-deletions (21 frameshift, 16 non-frameshift; 3.4%), as well as 12 known non-coding SNVs (1.0%) (**Figure 5-3A**). The majority of variants (71%) were present only in heterozygous form. Among exonic variants (**Figure 5-3B**), 25.9% of SNVs were common (ExAC MAF >5%), 14.2% occurred at a low frequency (ExAC MAF ≥1 and ≤5), whereas 59.9% were either rare or novel (ExAC MAF <1% or absent from ExAC or dbSNP137). MAFs in this study largely mirrored those reported in much larger data sets (ExAC, 1000G) (**Figure S5, Appendix E**. According to gene family or drug-related function, the *CYP* gene families had the most variants per targeted base pairs, followed by the UDP glucuronosyltransferases (*UGT)* and *ABC* family, then *SLC* family, while nuclear receptors were the least variable (**Figure 5-3C**). Individually, among Phase I enzymes, *CYP2D6* had the highest total number of SNVs (54) and the highest number of rare or novel variants from our gene panel (**Figure 5-3D**), and *UGT1A4* had the most SNVs among Phase II enzymes. Within the *SLC* family, organic cation transporter 1 (*SLC22A1*) showed the highest number of SNVs as well as rare or novel SNVs among all *SLC* genes sequenced. Among transporter genes of the *ABC* family, *ABCC2* had the highest number of the SNVs with 34 variants.

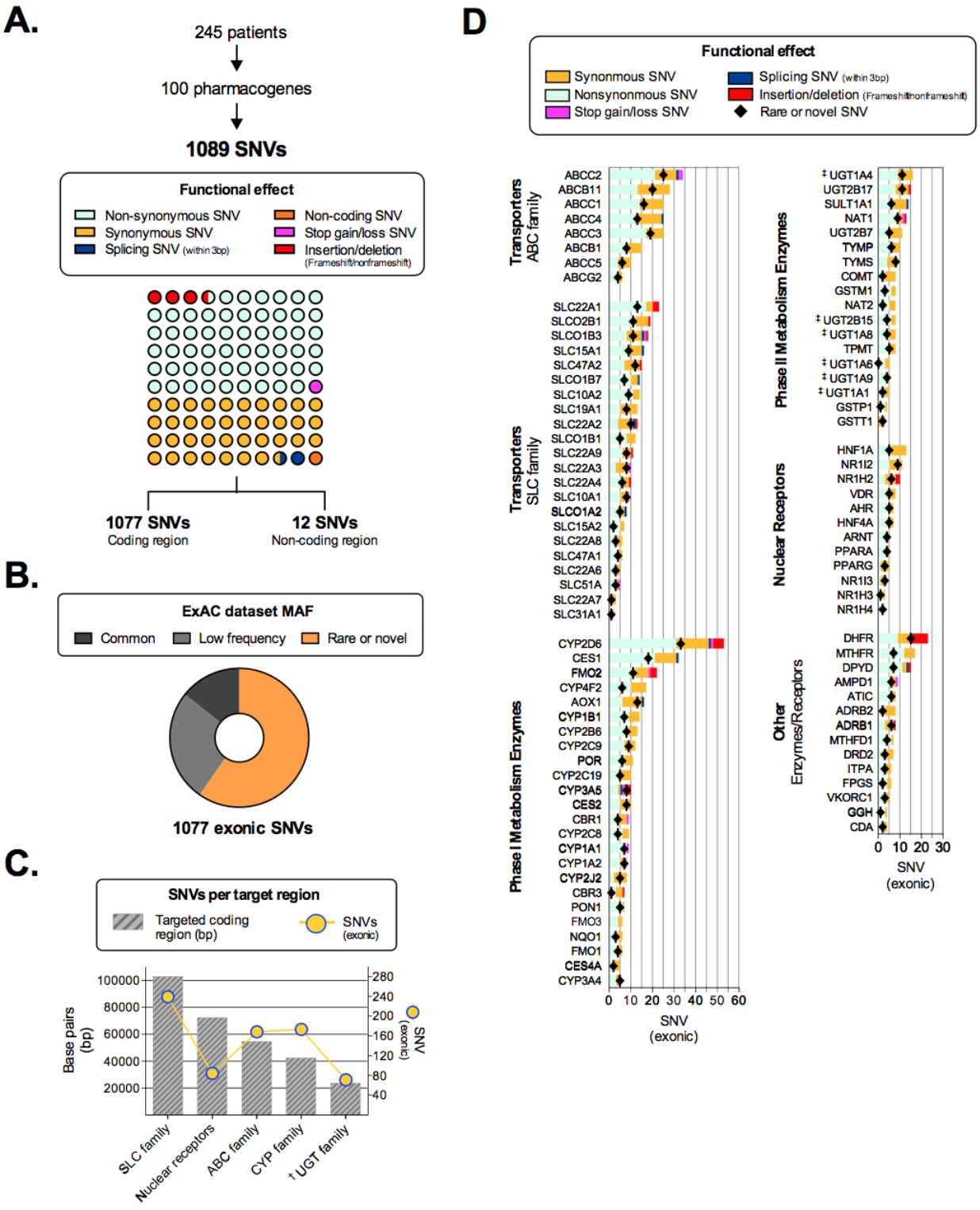
A.
245 patients
100 pharmacogenes
1089 SNVs
Functional effect
Non-synonymous SNV
Synonymous SNV
Splicing SNV (within 3bp)
Non-coding SNV
Stop gain/loss SNV
Insertion/deletion (Frameshift/nonframeshift)
1077 SNVs
Coding region
12 SNVs
Non-coding region
B.
ExAC dataset MAF
Common
Low frequency
Rare or novel
1077 exonic SNVs
C.
SNVs per target region
Targeted coding region (bp)
SNVs (exonic)
Base pairs (bp)
SNV (exonic)
SLC family
Nuclear receptors
ABC family
CYP family
†UGT family
D
Functional effect
Synonmous SNV
Nonsynonmous SNV
Stop gain/loss SNV
Splicing SNV (within 3bp)
Insertion/deletion (Frameshift/nonframeshift)
Rare or novel SNV
Transporters ABC family
Transporters SLC family
Phase I Metabolism Enzymes
Phase II Metabolism Enzymes
Nuclear Receptors
Other Enzymes/Receptors
SNV (exonic)

Figure 5-3. Analysis of variation in 245 subjects.

Analysis of genetic variation in 245 subjects assessed by PGxSeq. SNVs according to functional effect (A), allele frequency reported in ExAC database (B), number of exonic variants per target region (C), or gene (D). ‡ For UGT1A4, UGT1A6, UGT1A8, and UGT1A9, only SNVs located within the first exon were counted while shared exons were assessed only once with UGT1A1. ExAC, Exome Aggregation Consortium; MAF, minor allele frequency; SNV, single nucleotide variant.

5.3.3 *In silico* assessment of variants in pharmacogenes

Potential functional effects of the identified non-synonymous variants were assessed with CADD, PolyPhen-2, and SIFT. Our results showed marked differences between the prediction scores derived from these algorithms (**Figure 5-4**). However, the proportion of rare (MAF < 1%) or novel variants that were categorized as possibly deleterious was greater than the proportion of common (≥ 5%) or low frequency (≥ 1-5%) variants for all 3 tools (CADD: $p = 0.0004$, PolyPhen-2: $p = 0.0013$, and SIFT: $p = 0.0076$, **Figure S6, Appendix E**). The majority of pharmacogenes (87 out of 100) harbored at least one variant with a CADD score > 20 (median, 2). Rare or novel variants that were predicted as deleterious (CADD score > 20) were almost entirely observed in heterozygous form, with a median of two SNVs per subject (min-max, 0 - 8) across the 100 genes (**Figure S7, Appendix E**). Conversely, nearly all subjects (244 out of 245) had ≥ 1 common deleterious variant(s) in the homozygous form, median three (0 - 11) SNVs per subject. Finally, we assessed prediction scores among 12 CYP genes that account for the majority of known drug oxidation reactions (**Figure S7, Appendix E**); on average, 15.8% (5 - 95th percentile, 9.1 - 20.4%) of SNVs with a CADD > 20 among individuals were located within these gene.

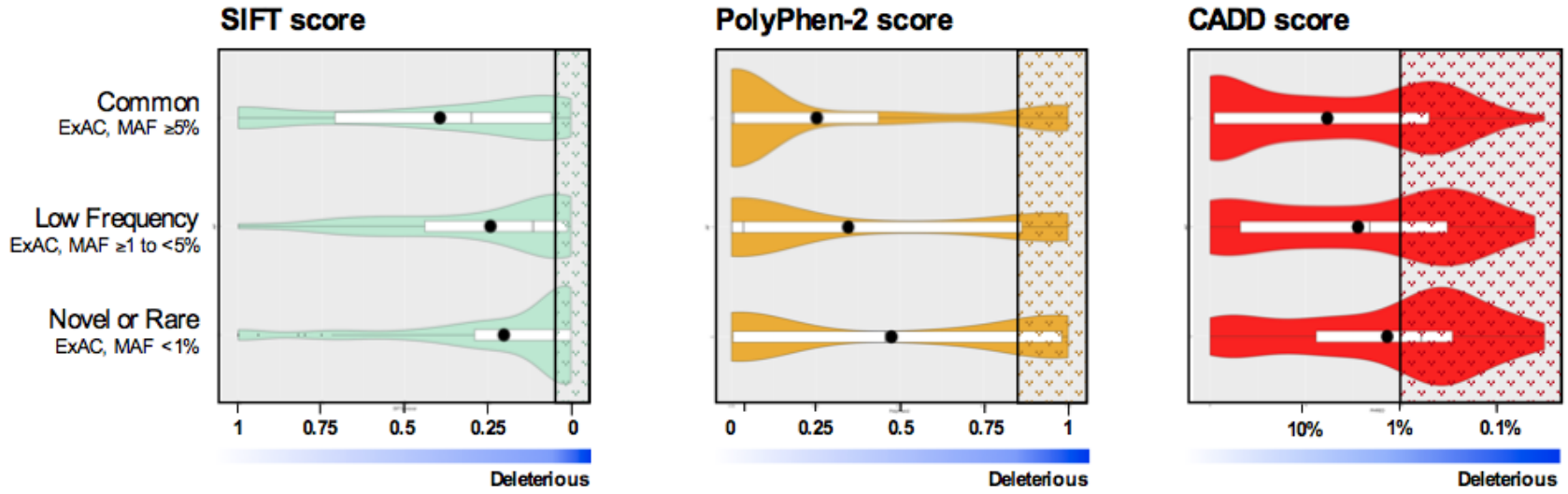

Figure 5-4. *In silico* assessment of non-synonymous variants in pharmacogenes.

In silico assessment of non-synonymous variants in pharmacogenes identified by PGxSeq (N = 245). (A) Frequency distribution of variants according to SIFT, PolyPhen-2, and CADD scores separated by minor allele frequency reported in the ExAC database. Shaded regions represent the proportion of potentially functional variants (or deleterious), defined as a scaled CADD score > 20 (11), a SIFT score < 0.05 (12), or a PolyPhen-2 score > 0.85 (13)

5.3.4 Variation in genes of clinical relevance

Among 9 clinically relevant genes for which Clinical Pharmacogenetics Implementation Consortium (CPIC) prescribing guidelines for specific variant-drug combinations have published, there are 29 "Level 1A/1B" variants categorized as having strong evidence for influencing drug efficacy/response and prescribing recommendations (https://cpicpgx.org/alleles/, accessed Dec 25, 2016). We identified 19 out of these 29 actionable variants in our cohort, with 161 patients (65.7%) harboring at least one CPIC level 1A/1B homozygous variant (**Figure S8, Appendix E**). We observed high read coverage in regions comprising CPIC Level 1A/1B variants as well as rare or novel SNVs with a CADD score >20 (**Figure S10, Appendix E),** illustrating the capacity of our gene panel for comprehensive genetic profiling of pharmacogenes with clinical relevance.

5.3.5 SNV discovery in apixaban AF cohort

We applied our panel in a small group of AF patients with either a high (n = 6) or normal (n = 6) apixaban plasma concentration phenotype (patient characteristics in **Table 5-3)**. Exonic and clinically relevant SNVs identified within the candidate genes selected for exploratory analysis among the high and normal apixaban concentration groups is shown **Table 5-4**. With the exception of a single variant within the *NR1I2* gene (rs149041795) belonging to an individual from the normal group, the remainder of nonsynonmous SNVs that were found in *ABCB1, ABCG2, CYP3A4,* and *CYP3A5* (no exonic variants were found in *NR1H4*) were common and not predicted to be deleterious according to *in silico* functional assessment tools. *ABCG2* c.421C>A and *CYP3A5*1* variants, have been reported to be associated with higher and lower apixaban plasma concentrations in Japanese

AF patients, respectively (16, 17). We observed a slightly greater frequency of the *ABCG2* c.421A allele in our high group compared to the normal group, and overall only one individual in our normal group was a carrier of the *CYP3A5*1* allele. We also identified individuals that were carriers for common functionally established variants found within our selected candidate genes. Two individuals within the high group were carriers of the *POR*28* variant. While within the normal group, one individual had a copy of the *CYP3A4*22* variant, and another individual had copy of the FXR/ *NR1H4* c.-1 G>T variant.

Table 5-3. Characteristics of apixaban-treated atrial fibrillation patients selected for sequencing.

Characteristic	Apixaban concentration	
	Normal	**High**
Subjects, n	6	6
Age, median (IQR)	68 (65-77)	70 (64-77)
Sex, Male (%)	100.0%	33.3%
Weight, kg, median (IQR)	106.6 (74.9-133.6)	95.0 (79.6-114.4)
Apixaban Dose		
5 mg twice daily, (%)	5 (100.0)	5 (83.3)
2.5 mg twice daily, (%)	0 (0)	1 (16.6)
Hours post dose, mean (min-max)	7.0 (6.3-7.8)	5.4 (3.2-8.9)
Apixaban plasma concentration		
ng/ml, median (IQR)	145.1 (120.2-155.8)	404.1 (260.8-445.3)
ng/ml/mg, median (IQR)	29.0 (24.0-31.2)	87.0 (70.3-93.0)
Serum Creatinine, μmol/L, median (IQR)	95.0 (70.0-105.0)	63.5 (51.8-82.0)
Creatinine Clearance, mL/min, median (IQR)	114.2 (78.16-183.2)	114.0 (95.8-132.3)

Table 5-4. Single nucleotide variants identified within high versus normal apixaban concentration groups.

Gene	Allele	Polymorphism	Type of SNV	dbSNP137	No. of algorithms predicting as damaging[a]	1000G (Euro) AF	ExAC AF	High group ‡ AF (n=6)		Normal group ‡ AF (n=6)	
ABCB1		c.2677 G>T	nonsynonymous	rs2032582	0/3	0.57	0.54		0.50		0.67
ABCB1		c.2677 G>A	nonsynonymous	rs2032582	0/3	NF	0.04		0.08		0.08
ABCB1		c.61 A>G	nonsynonymous	rs9282564	0/3	0.09	0.08		0.08		NF
ABCB1		c.3435 T>C	synonymous	rs1045642	(18, 19)†	0.47	0.5		0.50		0.58
ABCB1		c.2359 C>A	synonymous	rs200903110	-	NF	1.65E-05		0.08		NF
ABCB1		c.1236 T>C	synonymous	rs1128503	-	0.57	0.54		0.67		0.67
ABCB1		c.210 A>G	synonymous	rs2214102	-	0.9	0.94		0.92		0.83
ABCG2		c.421 C>A	nonsynonymous	rs2231142	(20, 21)†	0.1	0.12		0.25		0.17
CYP3A4		c.1242 C>G	synonymous	NF	-	NF	9.06E-05		0.08		NF
CYP3A4	*22	c.522-191 C>T	intronic	rs35599367	(22)†	0.05	NF		NF		0.08
CYP3A5		c.423 A>G	loss of stop codon	rs6977165	-	0.03	0.05		NF		0.08
CYP3A5	*3	g.6986 A>G	splice	rs776746	(23)†	0.95	NF		1.00		0.92
NR1H4		c.-1 G>T	5'UTR	rs56163822	(24)†	0.02	0.08		NF		0.08
NR1I2		c.1135 T>G	nonsynonymous	rs149041795	1/3	NF	2.00E-04		NF		0.08

NR1I2		c.879 T>C	synonymous	rs4058490	-	1	1		1.00		1.00
POR	*28	c.1508 C>T	nonsynonymous	rs1057868	(25) †	0.3	0.36		0.25		NF
POR		c.15 A>G	synonymous	rs10262966	-	0.01	0.07		NF		0.08
POR		c.387 A>G	synonymous	rs1135612	-	0.26	0.27		0.50		0.17
POR		c.1455 T>C	synonymous	rs2228104	-	0.97	0.92		1.00		0.92
POR		c.1716 G>A	synonymous	rs1057870	-	0.33	0.38		0.17		0.67

‡ Colours range represents allele frequencies: (□) not found or 0%, to 100% (■)

Abbreviations: AF, allele frequency; dbSNP 137, Single Nucleotide Polymorphism database build 137; ExAC, Exome Aggregation Consortium version 0.3; 1000G EUR, 1000 Genomes Project European dataset; NF, not found in dbSNP 137, 1000G EUR, ExAC database, respectively, apixban group. (†) References for known functionally established SNVs in bracklets. ([a]) Patient-specific SNV defined as damaging if scaled CADD score >20 **(11)**, a SIFT score <0.05 **(12)**, or a PolyPhen-2 score >0.85 **(13)**

5.4 Discussion

The principal findings of this study comprise: 1) the development of a comprehensive targeted NGS gene panel for most clinically important pharmacogenetic loci; 2) demonstrating the utility of this panel to simultaneously detect common and rare variants with minimal to no artefacts resulting from difficult to sequence regions; 3) demonstrating that PGxSeq performs at least as well as, and usually better than, current standard pharmacogenetic assays; 4) our exploratory analysis did not reveal likely deleterious SNV(s) within the selected candidate genes in the apixaban-treated AF subjects that were sequenced, that may potentially explain their observed elevated apixaban plasma concentration. Moreover, our results support the high prevalence of rare or novel variants of potential functional relevance within pharmacogenes.

Compared to traditional genotyping or sequencing strategies, the applied targeted exome sequencing strategy enabled the comprehensive discovery of novel rare SNVs as well as accurate genotyping for common, previously established functional variation across exonic and intergenic regions in clinically important pharmacogenes with fast and adequate performance. Available bioinformatics tools further allowed customized utilization of sequencing data at a small or large scale, i.e. the assessment of individual genotypes and genes of interest or a more exhaustive pharmacogenetic analysis. Our target sequence included 29 clinically actionable variants with recommendations to adjust dose or for alternative therapies. Nearly all patients (97%) harboured one or more CPIC Level 1A/1B variant(s) confirming recent findings from the eMerge-PGx study comprising extensive sequencing data

from 5000 patients for 82 pharmacogenes (26). The multitude of other, newly discovered candidate variants among CPIC genes in this study highlights the need for comprehensive sequencing approaches to determine the likely more complex genotype of a patient, while high-throughput experimental strategies are warranted to screen and confirm effects of previously unreported genetic variation on protein activity.

A significant number of pharmacogenetic variants detected in our total cohort was either rare or novel (60%), and more than half (56%) resulted in amino acid changes, supporting previous observations in larger datasets (1, 26-28). While the proportion of SNVs with predicted effects on protein function differed among applied *in silico* tools, differences in scoring have been previously observed and are not surprising given the way these algorithms were derived (29). SIFT leverages the evolutionary conservation of amino acids (12), PolyPhen-2 uses pathogenicity information (13), while CADD is the most recent algorithm integrating conservation metrics, regulatory information, and protein-level effect among others (11). A recent study suggests that the *in silico* algorithms used here predict altered enzymatic or transporter function with about 80% accuracy compared to *in vitro* assessment (29). Among the possibly deleterious variants identified, rare or novel SNVs were more likely to have functional effects than common or low frequency variants (**Figure S6, Appendix E**), with 43.1 – 53.5 % of rare or novel SNV predicted to be deleterious according to *in silico* algorithms. These findings are similar to a recent report evaluating NGS data from thousands of individuals in 146 pharmacogenes, where 30% to 40% of rare variation was predicted to be functional (1). Moreover, we found

that 94% of patients (231 of 246) carried at least one deleterious allele (CADD score >20) in 12 *CYP* genes with key roles in drug metabolism (27, 30); these potentially clinically relevant findings need to be followed up.

In this study, accurate variant and genotype calls were obtained in pharmacogenes previously reported to be structurally problematic due to sequence similarities but also due to highly variable or repetitive regions (29, 31-34). NGS-derived genotypes were concordant with TaqMan results for all the common and/or clinically relevant SNP that were tested. In addition, retrospective Sanger sequencing confirmed 5 of the 5 rare variants identified by NGS in a subset of relevant genes.

Genetic profiling using any short-fragment sequencing platform is a widely recognized challenge for NGS of pharmacogenes (31, 35), and requires sufficient representation of mapped sequenced reads in the region of interest to ensure accuracy. As expected, many members of the *CYP*, *SULT* and *UGT* gene families were reported as harbouring 250-bp sequence fragments that map to more than one place in the genome due to their sequence similarity, with regions that are up to 100% identical (i.e. pseudogenes) predicted of being the most problematic (36). An estimated 1.8% of our 422kb target sequence (69 exons in 19 genes) was found to be susceptible to potential mismapping. Although our hybridization-based enrichment strategy achieved a mean read coverage above 80x for most genes (98 of 100), the results also indicate that the mean value alone may not always correctly indicate sufficiently even read coverage across the targeted coding region. Specifically, for *CES1* a mean DOC $\geq 80\times$ was observed, however 30.2% of its targeted bases (Exon 12 - 14) showed a DOC $<30\times$ indicating areas prone to higher

error rates (**Figure 5-2**, **Figure S3** **Appendix E**); a 95 - 100% sequence similarity has been previously reported for *CES1* exons 12 - 14 (36). Accordingly, high homology regions may benefit from longer capture probes for hybridization-based target enrichment to ensure appropriate capture and/or sequence read mapping. Moreover, DOC for *GSTM1* and *GSTT1* were the lowest among all genes of our panel. A previous report in a Korean population sample showed individuals with *GST* gene deletion (GST*0) lacked coverage when assessed with NGS, while the number of gene copies correlated the mean number of sequencing reads (29). Deletions of *GSTs* are also prevalent among Europeans (MAF ~ 0.5 (37)), and we noted 52% and 21% of our study group had near zero coverage for *GSTM1* and *GSTT1*, respectively, likely representing GST*0 carrier status (**Figure S9**, **Appendix E**). While NGS is thought to be best suited for the detection of SNV, our findings and most recent reports suggest that a read-depth based approach may be also used for the identification of CNV as demonstrated for *GSTs* (29), the LDL receptor (*LDLR*) (38) and various genes underlying retinal dystrophies (39) using gene panels, as well as *CYP2D6* utilizing whole-genome sequencing data (33). Our findings highlight the need for monitoring targeted regions for low sequence coverage, absent data or ambiguous calls to reduce false negative or positive findings by defining test panel limitations in agreement with current clinical laboratory standards for NGS (40).

Utilizing existing knowledge of apixaban's metabolism and disposition, we took a candidate gene approach to explore new, previously not recognized single nucleotide variations that potentially correlate with apixaban concentration

phenotype in a small subgroup of AF patients. While our sample size is too small to draw any statistical inferences, we compared the variants identified between subjects with high and normal concentration phenotypes using descriptive statistics. We did not find rare or patient-specific deleterious SNV(s) within the seven candidate genes in our subjects belonging to the high group that may potentially explain their observed elevated apixaban plasma concentration. Furthermore, the distribution of common clinically relevant SNVs identified between the high *versus* normal concentration group were not always conceptually consistent with the known functional effect of each variant. For instance, the *POR*28* variant, which has been reported to be associated with increased *CYP3A* activity (25), was observed only in the high group (1 heterozygous, 1 homozygous). Theoretically, this variant would be protective against higher apixaban plasma concentration. On the other hand, we observed a higher frequency of the reduced function variant *ABCG2* c.421 C>A within the high concentration group compared to the normal group. This observation is consistent with the high concentration phenotype, the *ABCG2* gene codes for the transporter, breast cancer resistance protein (BCRP), which plays a role in limiting the absorption of apixaban in the gut (41). It is also worth noting that, even though the *in silico* prediction tools that were used focus on the impact of nonsynonymous SNVs, we cannot rule out the influence of synonymous or non-coding variants (intronic, 5' or 3' untranslated regions). A classic example of this is the synonymous *ABCB1* c.3435C>T variant which has been implicated in altering the pharmacokinetics and pharmacodynamics of a number of drugs (19, 42).

While we show the potential application of targeted exome sequencing as a comprehensive pharmacogenetic profiling tool, there are some limitations. Validation of concordance was limited to variants in 41 loci in 21 genes in our relatively small, mostly Caucasian sample, in contrast to previously reported multi-center studies that assessed hundreds of SNVs in larger populations (29, 32) including commercially available DNA control samples (29, 40). At present, despite high concordance rates for common *CYP2D6* and *CYP2C9* variants, we cannot exclude false-positive variant calls. We intend to further evaluate our panel considering intronic SNVs and CNVs for *CYP2D6* in a larger, previously characterized patient sample. Despite the small sample size, the herein observed variation compared well to findings from larger data sets.

Next-generation sequencing platforms are starting to impact upon many clinical fields, especially cancer and pediatrics. Bringing these technologies to clinical pharmacogenetics represents a timely and logical convergence, especially given the history of applied genetic concepts and molecular methods within the discipline. Through comprehensive validation of performance and accuracy, results from our study and others demonstrate the utility of targeted exome sequencing panels as sensitive and reliable sequencing platforms for pharmacogenes (29, 32). But despite the relative ease of the sequencing process, the time and effort required for post-sequencing computational and bioinformatics data analyses are significant due to the technical and interpretive complexity of NGS and the biology of some pharmacogenetic gene targets. Moreover, as new variants are discovered using these high-throughput detection methods, the need for standards in attributing

pathogenicity together with development of tools for high-throughput functional assessment and clinical validation are required before implementing findings to aid therapeutic decision-making.

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6 Discussion and conclusion

6.1 Summary and Discussion

6.1.1 Chapter Three

Factor Xa inhibitors (FXaIs), rivaroxaban and apixaban, represent a new class of oral anticoagulants that are widely prescribed as an alternative to warfarin therapy for stroke prevention in patients with atrial fibrillation (AF). An important advantage of these drugs is that routine monitoring of anticoagulation response is not necessary. Nevertheless, because of their mechanism of action, FXaI pharmacological effect can be inferred based on the observed plasma drug concentration, yet there is a paucity of data relating to variation in FXaI concentration among patients taking these drugs in the post-market clinical setting. Therefore, the aim of Chapter Three was to measure the extent of interpatient variation in the circulating FXaI plasma concentrations of 243 atrial fibrillation patients prescribed rivaroxaban and apixaban within a routine clinical care setting, and we hypothesized that this variation was greater than that observed in clinical trials. In our routine-care setting rivaroxaban and apixaban plasma concentrations tended to be more variable than those observed in clinical trials, with approximately 12% of patients receiving rivaroxaban and 13% of patients receiving apixaban exceeding the 95th percentile for the predicted maximum plasma concentration observed in clinical trials. These results supported our hypothesis, and accordingly we set out to identify additional clinical and molecular determinants that more fully predict patients at risk for excessively high or low FXaI concentration.

6.1.2 Chapter Four

Renal function, age, sex, weight, as well as carrier status for functional genetic polymorphisms within genes that code for proteins involved in the transport (ATP binding cassette [ABC] G2 gene) and metabolism (cytochrome P450 [CYP] 3A5 gene) of apixaban have been demonstrated to impact the elimination of the drug in humans as indicated by altered apixaban pharmacokinetics, and ultimately drug exposure (1-4). Furthermore, as part of its metabolism, apixaban is primarily oxidized by CYP3A enzymes (5, 6). However, the relationship between apixaban blood concentrations and endogenous markers of CYP3A activity, as a predictor for interindividual variability of apixaban exposure, has not been determined. Accordingly, the aim of this chapter was to examine 4β-hydroxycholesterol (4β-OHC) (as a marker for apixaban metabolism) along with the factors that are known to impact apixaban exposure such as renal function, age, sex, weight, and common genetic variation, in order to better explain the variation in concentration observed within our apixaban-treated cohort. We hypothesized that 4β-OHC concentration with clinical and genetic variables would better explain the variation in FXaI concentration compared to clinical and genetic factors alone. We found a weak but statistically significant correlation between plasma concentrations of apixaban and 4β-OHC, with higher 4β-OHC concentrations (higher CYP3A4 activity) associated with lower apixaban concentrations. Moreover, multivariable linear regression analysis demonstrated that 4β-OHC concentration was an independent predictor of apixaban concentration in our cohort along with age, female sex, serum creatinine, and amiodarone use. Weight, diltiazem use, as well as *CYP3A4*22*, *CYP3A5*3*,

ABCB1 c.3435C>T, or *ABCG2 c.421C>A* carrier status were not significant predictors of apixaban plasma concentration in our cohort. Although our findings supported our hypothesis, we accounted for only 27.9% of the interindividual variability in FXaI plasma concentration observed in our apixaban-treated AF cohort (n = 119), leaving the majority of the variation unaccounted for.

6.1.3 Chapter Five

Clinical variables, common genetic variation, and 4β-hydroxycholesterol concentration accounted for 31% of the interindividual variability in FXaI plasma concentration observed in our apixaban-treated AF cohort (n = 119), leaving the majority of the variation unaccounted for. Therefore, we hypothesized that part of this unexplained variation was the result of certain individuals harbouring rare or patient-specific functional single nucleotide variants (SNV) within candidate genes encoding drug transporters and enzymes relevant to apixaban's disposition. Recent large-scale whole genome or exome sequencing studies have revealed that humans harbor a large number of rare, and/or potentially deleterious variants in genes relevant to drug disposition and response (7, 8). Targeted next-generation sequencing (NGS) represents a new time- and cost-effective technology for detecting common and rare genetic variation. In Chapter Five, we aimed to develop a targeted sequencing approach to detect common and rare genetic variation in pharmacogenes pertinent to drug disposition and response. We subsequently, applied this method for single nucleotide variant discovery in a subgroup of apixaban-treated AF patients with aberrantly high apixaban plasma concentrations compared to controls. Overall.

we were able to generate a sufficient depth-of-coverage (DOC) >30x for 97.4% of the target sequence, and for a select number of SNVs (32 common and 5 rare or novel) we confirmed concordance between genotypes determined by NGS *versus* TaqMan genotyping or Sanger sequencing. More specifically, our exploratory analysis did not reveal likely deleterious SNV(s) within the selected candidate genes in the apixaban-treated AF subjects that were sequenced, which may potentially explain their observed elevated apixaban plasma concentration. Although this did not support our hypothesis, at this point we cannot rule out the influence of synonymous or non-coding variants (located in intronic, 5 or 3 prime untranslated regions), as well as structural variants (copy number variations or large insertion and deletions), as these variations could also have influence over gene expression, and in turn the exposure of apixaban in these individuals. We were also limited by a small sample size, sequencing a larger cohort would have allowed us to test for a difference in the burden of SNVs within these candidate genes between our high and control group.

6.2 Therapeutic implications

Since their approval, the use of direct-acting oral anticoagulants (DOACs), such as apixaban and rivaroxban, for stroke prevention in patients with AF has rapidly increased (9, 10), owing to their ease of prescribing and management. While warfarin prescribing is complicated by routine INR testing and high interpatient variability in the required therapeutic dosage (20-fold), DOACs offer fixed dosing (up to 2-fold) without the need for routine monitoring. Although DOACs appeared to have a

favourable risk-benefit profile in clinical trials compared to warfarin (11), major bleeding is still a complication associated with DOAC use. Moreover, the patient population that take DOACs in the post-market setting may vary greatly with their co-morbidities and extensive co-medications from those within clinical trials. As prescribing trends in clinical practice continue to favour the use of DOACs, the number of bleeding events associated with them is likely to increase as well. Identifying factors that increase the DOAC-associated bleeding risk in patients before prescribing these drugs can help to mitigate adverse drug events. Aside from non-DOAC related factors that increase bleeding risk such as advanced age, pharmacodynamic interactions from co-medications, and medical conditions or procedures with a pre-existing risk for hemorrhage; prolonged elevation of DOAC plasma concentrations will exacerbate the risk of bleeding associated with the use of these medications (12-15).

As observed in Chapter Three, when dosing according to clinical guidelines, the measured FXaI plasma concentrations for a subgroup of AF patients taking apixaban and rivaroxaban within a routine care setting will likely fall in the extremes of FXaI drug exposure. Although, our study was not designed to identify an association between FXaI concentration and bleeding or stroke events, this relationship has been described in the product monograph of apixaban (12), rivaroxaban (15), and more recently published for edoxaban (a novel FXaI inhibitor) (16). However, without an established threshold for efficacy (thrombosis) or safety (bleeding), such as the one established for warfarin with INR, making dose adjustments or constructing dose algorithms based on observed FXaI concentrations will remain to be clinically

challenging. Ultimately, our findings provide support for a need to establish a therapeutic range of FXaI plasma concentrations for which the risk of bleeding and stroke are at the lowest. On the other hand, physicians can currently use FXaI plasma concentration as a means to ensure patient compliance, especially among those patients experiencing stroke or transient ischemic attacks while prescribed rivaroxaban and apixaban.

In Chapter Four, we attempted to characterize the inter-individual variability in the FXaI plasma concentrations observed in our apixaban-treated AF cohort from Chapter Three. We found that the majority of the explainable variation in apixaban concentration was accounted for by age and renal function which are currently assessed as part of apixaban dose selection. In addition to this, our results demonstrated that measuring endogenous biomarkers of CYP3A4 activity such as 4β-hydroxycholesterol may help determine whether an unexpectedly low apixaban plasma concentration is the result of high CYP3A4 activity. This can provide physicians with an additional layer of evidence to support increasing apixaban dosage or a change in medication to one that is independent of CYP3A4 metabolism. Concomitant use of amiodarone was associated with higher apixaban plasma concentration in our AF cohort. Patients who experience bleeding while taking amiodarone with apixaban, may benefit from therapeutic drug monitoring as a method for dose adjustment, especially if they have elevated apixaban concentrations.

6.3 Future direction

Future prospective studies involving AF patients within routine care that focus on rivaroxaban and apixaban exposure in relation to clinically relevant outcomes (major bleeding, stroke, death) are needed to determine the FXaI concentration thresholds for safety (bleeding) and efficacy (stroke). Not only will this help elucidate the true clinical significance of the observed variation in FXaI plasma concentration that was found in our studies, but it will also accelerate the field of research involved in characterizing FXaI interpatient variation.

The study we carried out in Chapter three was observational, involving patients who were recruited during their routine care visits. As such, we were limited in our ability to account for the effect of adherence as well as inaccurate self-reporting of time of last-dose on the drug concentration. Therefore, it remains possible that unreported missed doses or drug intake without food (rivaroxaban only) may explain some of the inter-patient variability especially in patients with very low plasma concentrations. Prospective studies, that ensure both patient adherence to dosing and sampling of blood to obtain either trough FXaI plasma concentrations or an area-underneath-the-curve, would likely reduce the proportion of unexplained variation.

Recent work carried out in a Japanese AF population have reported the *ABCG2* 421A/A and *CYP3A5**1/*1 genotype to be associated with higher and lower trough apixaban concentrations, respectively (4, 17). While we did not observe an association between these functional polymorphisms and variability in apixaban plasma concentration, this may in part be due to the fact that there weren't many

variant carriers in our cohort. Further studies in larger AF cohorts including more variant carriers from different ancestries will help confirm the effect of these polymorphisms on apixaban plasma concentration, while *in vitro* and animal studies characterizing the molecular mechanism behind the altered plasma concentration (i.e., decreased absorption and/or reduced excretion) would strengthen Ueshima et al., observations in human. For instance, *in vitro* experiments examining the difference in the vectorial transport of apixaban within human intestinal and kidney cell models expressing wildtype ABCG2 (breast cancer resistance protein, BCRP) or the c.421C>A variant.

Through developing our targeted NGS panel (Chapter Five) we discovered the majority of individuals that were sequenced had some degree of burden in terms of deleterious SNVs across their pharmacogenes. These variants ranged from clinically actionable SNVs, with a strong evidence for influencing drug efficacy/response, to newly discovered candidate SNVs that were predicted to be deleterious according to *in silico* functional assessment tools. The multitude of variants detected in each individual highlights the need for comprehensive sequencing approaches to determine the likely more complex genotype of a patient. But despite the relative ease of the sequencing process, the time and effort required for post-sequencing computational and bioinformatics data analyses are significant; complicated by the technical and interpretive complexity of NGS and the biology of some pharmacogenes. One of the areas that will benefit from developing more integrated software for post-sequencing data extraction is the identification of copy number variations from NGS data, including partial gene insertions and deletions –

eventually becoming a growing area of research as it pertains to genotyping (18-20). Additionally, the field of pharmacogenetics will likely benefit from *in silico* prediction tools that tailor the impact of an amino acid change in terms of its influence on drug-protein substrate specificity for a drug of question – perhaps by incorporating knowledge of the regio-specific interactions between drug substrates and the relevant proteins involved in their disposition pathway. This will assist in filtering worthwhile candidate variants for investigation in those FXaI-treated AF subjects who demonstrate unexpectedly high or low drug plasma concentrations. Ultimately, as variants are discovered using these high-throughput detection methods, the need for standards in attributing pathogenicity together with development of tools for high-throughput functional assessment and clinical validation are required before implementing findings to aid therapeutic decision-making.

6.4 Conclusion

Today, FXaIs are prescribed at fixed doses without the need to assess the degree of anticoagulation. As prescribing trends in clinical practice continue to favour the use of FXaIs for preventing cardioembolic stroke in patients with atrial fibrillation, the number of adverse events associated with these drugs is likely to increase as well. The identification of factors that drive variation in FXaI exposure and response will provide the basis for a safe, yet efficacious approach to dose selection. The studies presented within this thesis provide insight into the factors that drive variation in

FXaI concentration, and may serve as a framework for future investigations regarding personalizing FXaI anticoagulation therapy.

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7 Appendices

Appendix A

Office of Research Ethics
The University of Western Ontario
[redacted]
Telephone: [redacted]
Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R.B. Kim
Review Number: **15586** **Review Level:** Full Board
Review Date: October 21, 2008
Protocol Title: Pharmacogenetics and drug response
Department and Institution: Medicine-Dept of, London Health Sciences Centre
Sponsor:
Ethics Approval Date: December 09, 2008 **Expiry Date:** November 30, 2012
Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent (v.6 Nov 2008)
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

[redacted] Chair of HSREB: Dr. Joseph Gilbert

Ethics Officer to Contact for Further Information			
☐ Janice Sutherland	☐ Elizabeth Wambolt	☐ Grace Kelly	☒ Denise Grafton

[redacted]

This is an official document. Please retain the original in your files.

cc: ORE File
LHRI

Date: 18 October 2017

To: Richard Kim

Project ID: 5683

Study Title: Pharmacogenetics and drug response (REB# 15586)

Application Type: Continuing Ethics Review (CER) Form

Review Type: Delegated

FB Reporting Date: November 7, 2017

Date Approval Issued: 18/Oct/2017 11:31

REB Approval Expiry Date: 25/Nov/2018

Dear Richard Kim ,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

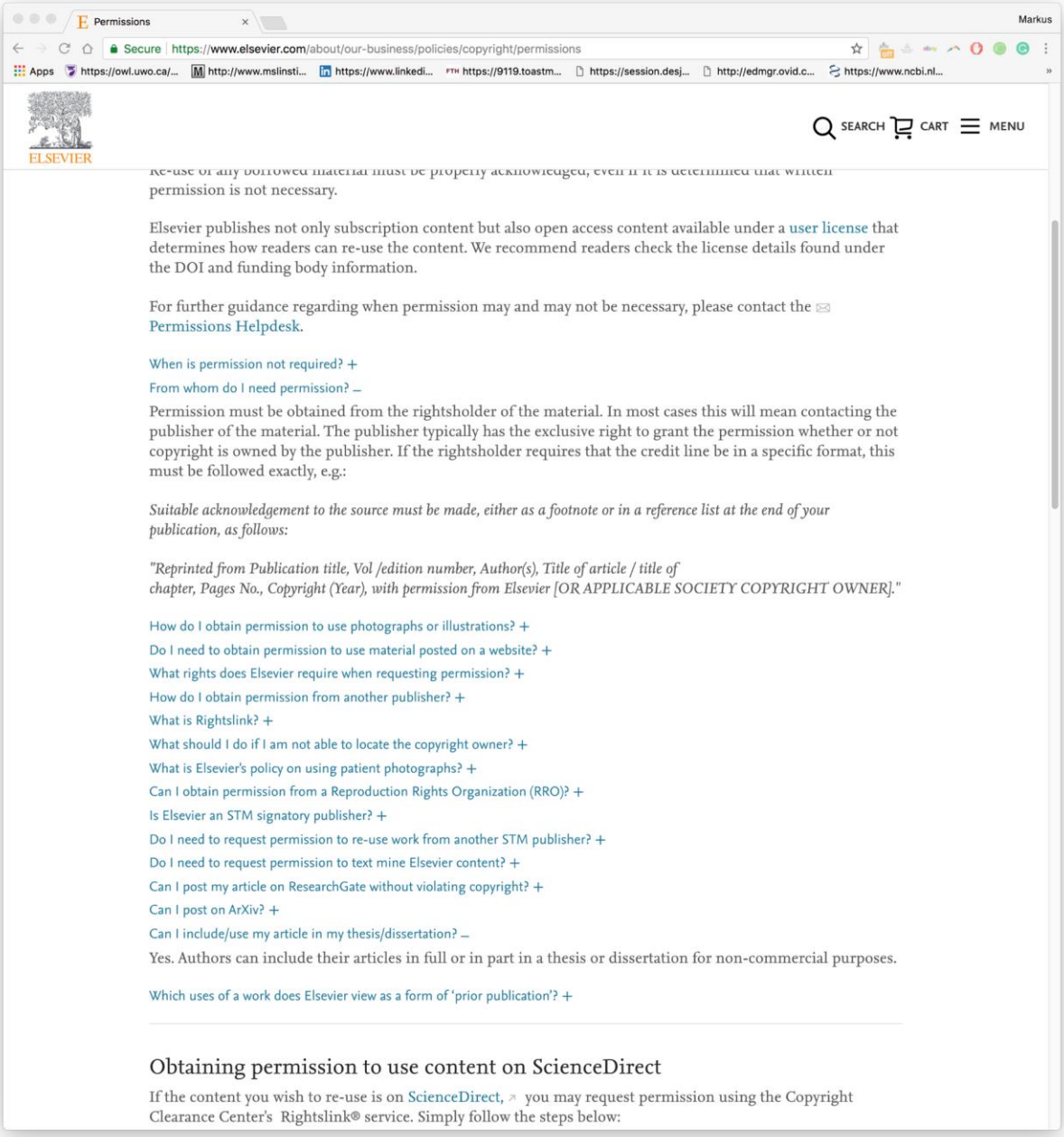
Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Kelly Patterson

Appendix B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Country: Canada
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Title: Student
Lab/Department: Clinical Pharmacology
[redacted]
Title of Publication: PhD Thesis
Authors/Editors: Markus Gulilat, Supervisor: Richard B. Kim
Date of Publication: Jan 2019
Publisher: Western Univerisity
Title of CSHLP Journal/Book: Next-Generation Sequencing in Medicine
Title of Article/Chapter: The Role of Next-Generation Sequencing in Pharmacogenetics and Pharmacogenomics
CSHL Authors/Editors: Ute I. Schwarz, Markus Gulilat, Richard B. Kim
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Figure Numbers: Figure 1
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Appendix C

Multiple linear regression coefficients for apixaban plasma concentration in atrial fibrillation patients (n = 119).

Predictor variable	**B**	**95% CI**	***P*-value**
Dose	0.109	0.073, 0.146	<0.001
Hours post dose	-0.016	-0.035, 0.002	0.086
Age, years	0.008	0.004, 0.012	<0.001
Sex, Female	0.069	-0.006, 0.144	0.069
Weight, kg	<0.001	-0.002, 0.002	0.985
Serum creatinine, µmol/L	0.002	0.001, 0.003	<0.001
ABCB1 c.3435C>T , per allele	0.001	-0.049, 0.051	0.972
ABCG2 c.421C>A, 1 or 2 allele	0.063	-0.018, 0.144	0.125
CYP3A4*22, 1 or 2 allele	0.027	-0.069, 0.124	0.574
CYP3A5*3, 1 or 2 allele	0.002	-0.111, 0.116	0.971
Amiodarone use	0.128	0.027, 0.230	0.014
Diltiazem use	0.015	-0.080, 0.110	0.756
Adjusted *R* squared 0.235.			

Appendix D

Detailed methods

Sample collection

Genomic DNA (gDNA) was obtained from venous blood samples of 245 Caucasian subjects following written informed consent. We initially evaluated sequencing performance and accuracy of variant detection in the first 48 subjects − this was our validation cohort. Following this, all 245 subjects that were sequenced were used to evaluate genetic variation in terms of population allele frequencies and predicted functional effect. Then we carried out candidate gene SNV discovery in a subgroup of 12 AF patients with a known apixaban drug concentration phenotype. This study was approved by the Research Ethics Board of Western University, London, Canada.

Gene selection, capture probe design and enrichment method

We used the Nextera Rapid Capture Custom Enrichment Kit (Illumina, San Diego, CA) for the enrichment of genomic regions of 100 genes encoding major cytochrome P450 (CYP) enzymes, phase II conjugation enzymes, drug transporters of the solute carrier (SLC) and ATP binding cassette (ABC) families as well as regulatory proteins of relevance to variability in drug ADME (absorption, distribution, metabolism, excretion) and response. In addition, regions encompassing 14 known functional promoter or intronic SNVs were included (Table 1).

Table S1. Promoter and intronic SNVs included in probe design.

	Gene	Allele	dbSNP137	Position[a]
Promoter regions	CYP1A2	*1C	rs2069514	Chr15:75038220
	CYP2C19	*17	rs12248560	Chr10:96521657
	UGT1A1	*28	rs8175347	Chr2:234668881: 234668882
	UGT1A1	*60	rs4124874	Chr2:233757013
	VDR		rs11568820	Chr12:47908762
	VDR		rs4516035	Chr12:47906043
	VKORC1		rs9923231	Chr16:31096368
	NR1H4		rs56163822	Chr12:100493323
Intronic regions	CYP1A2	*1F	rs762551	Chr15:74749576
	CYP3A4	*22	rs35599367	Chr7:99768693
	VKORC1		rs9934438	Chr16:31093557
	CYP3A5	*3	rs776746	Chr7:99672916
	CYP2D6	*41	rs28371725	Chr22:42127803
	NR1I2		rs2276707	Chr3:119815306

([a]) Chromosome position based on the GRCh37 assembly

A total of 10,207 capture probes (80 bp) were custom-designed using the Illumina Design Studio (Illumina, San Diego, CA) comprising 722 kilobases (kb) of sequence per sample (Genomic coordinates can be found in Table S1, Supplemental Digital Content 1). Exons of all coding isoforms were targeted for selected genes including 300-bp intronic (flanking each exon) and 250 bp of 5' and 3' untranslated regions (UTR). Chromosomal coordinates were obtained from University of California Santa Cruz (UCSC) genome browser using the GRCh37/hg19 human genome assembly. DNA library preparation and subsequent target-capture sequencing was conducted at the London Regional Genomics Center, London, Ontario, as previously described (1). Briefly, DNA samples were processed in runs in batches of 12 or 24 samples. After serial dilutions, DNA was adjusted to a final concentration of 5.0 ± 1 ng/µl using the Qubit DNA kit (Invitrogen, Eugene, OR). DNA was enzymatically fragmented, polymerase chain reaction (PCR)-amplified with individual sample barcodes, equimolar pooled, hybridized to the biotinylated capture probes, pooled using streptavidin beads, and PCR-amplified again to select the final target sequence. Resulting libraries were quantified, and loaded on to a standard flow-cell on the Illumina MiSeq Sequencer (Illumina, San Diego, CA) using 2 x 300 bp or 2 x 150 bp paired-end chemistry.

Base calling, sequence alignment and variant detection

Prior to the alignment of reads to the reference genome, sequencing performance metrics were assessed. For each run, cluster density (1000/mm^2), total number of reads passing instrument filters and per base quality score (also Phred score, Q;

describes the probability of a sequencing error as a measure of base call accuracy) was obtained. Paired-end sequenced reads were separated according to sample-specific barcodes and sequencing data downloaded as FASTQ files that were further assessed with the quality control tool, FastQC (2), including base quality across reads, per sequence GC content, sequence length distribution and duplication level (3).

Alignment of sequencing reads and variant calling were performed using the CLC Bio Genomics Workbench 7.0 (CLC Bio, Aarhus, Denmark) through a custom-automated workflow. FASTQ files were imported and mapped to the reference human genome (GRCh37/hg19 build). Using default algorithms (i.e. Local Realignment and Remove Duplicate Mapped Reads), initial read mapping was further optimized around insertion-deletion mutations (indels) and PCR duplicates removed. To further ensure accuracy of variant and genotype calling, quality-based variant detection tools were employed with the following parameters: diploid organism, probability of non-reference allele ≥ 95% (versus sequencing error), ≥ 10-fold coverage (10 x), ≥ 30% read frequency, and ≥ 30 per base quality score at the variant location. Resulting sequence variation reports were exported in variant call format (VCF) for downstream annotation.

Coverage (also read depth) or depth-of-coverage (DOC) was defined as the number of reads mapped to a genomic position following alignment of sequenced reads and removal of duplicate reads. Reads that were non-specific matches (mapped to more than one location of hg19 reference genome) or missing the paired read were excluded from this calculation. For every subject, a Coverage Summary Report

along with a base-by-base Coverage Table were exported. Coverage analysis was restricted to coding regions, including 10 bp before and after each exon, and 250 bp of 3' and 5'UTR. We assessed inter-subject and intra-subject variability of DOC across target sequence by sequencing cluster (n = 12 or 24), and the mean (±SD) for each gene. As an additional indicator of low coverage regions within a gene we expressed DOC as the percentage of targeted bases having mean DOC < 30 ×.

Variant annotation and *in silico* functional assessment

Functional annotation of SNVs was carried out using ANNOVAR (4) through *in silico* prediction algorithms such as Combined Annotation Dependent Depletion (CADD) (5), Sorting Intolerant from Tolerant (SIFT) (6), and PolyPhen-2 (7), and variant frequency among different populations were determined utilizing large genomic databases (RefSeq, dbSNP137, 1000 Genomes, Exome Aggregation Consortium [ExAC]; obtained July 8, 2015). SNVs with a CADD Phred score (scaled) greater than 20 (5), a SIFT score of less than 0.05 (6), or a PolyPhen-2 score of greater than 0.85 (7) were considered as potentially functional variants (altering protein function), and herein defined as " deleterious". Variants were classified as 1) non-synonymous (coding variants resulting in amino acid change), 2) synonymous (coding variants without amino acid changes), 3) frameshift deletion or insertion (in-del), 4) splicing (3 nucleotides within an intron-exon boundary), 5) stop gain or loss, or 6) functional intronic or promoter variants. Coding variants were further grouped by MAF reported in the ExAC database as common (MAF ≥ 5%), low frequency (1% ≤ MAF > 5%), or the combined category of rare (MAF < 1%) and novel (absent

from ExAC and dbSNP build 137 databases). *In silico* functional assessment was restricted to protein-coding genetic variation and gain or loss of a stop codon.

Concordance assessment

To assess concordance of NGS results, orthogonal genotyping was performed using TaqMan allelic discrimination for 32 common functionally validated or clinically relevant SNVs. Rare NGS variants were confirmed retrospectively by Sanger sequencing within 7 pharmacogenes. Polymerase chain reaction conditions and primers for Sanger sequencing and TaqMan assay IDs are listed in Table 2, and Table 3 respectively.

Table S2. Polymerase chain reaction conditions and primers used for Sanger sequencing of rare NGS variants.

Abbreviations: dbSNP137, Single Nucleotide Polymorphism database build 137; PCR, polymerase chain reaction; FOR, forward primer; REV, reverse primer.

Gene	dbSNP137/Position[a]	PCR Target		Primer set		Product Size (bp)	Ref.
		Exon	RefSeq	(5' to 3')			
ABCB1	rs55852620	(24/26)	NM_000927	FOR	GAGAATTAATCTATGTGATTATGG-	263	(8)
				REV	GTATTTAACATCTCATACAGTCAGAG		
CYP2D6[b]	rs5030867	(6/9)	NM_000106	FOR	GCTAACTGAGCACA-	577	(9)
				REV	CCGGCCCTGACACTCCTTCT		
SLCO1B1	Chr12:21350002	(8/15)	NM_006446	FOR	AATCTTACATGACTTACGTTCAC-	242	(10)
				REV	CCACTTGGAATACAGTATTTAG		
SLCO1B1	Chr12:21355448	(10/15)	NM_006446	FOR	TCTGCTTTCACTTTACTTCTTCC-	195	(10)
				REV	GAATAAGGAGAGGAAAGTAAAAAC		
SLCO1B3	Chr12:21011433	(3/14)	NM_001009562	FOR	GGGCATTCAGTTCTACTAGA-	233	(11)
				REV	TAATAAATGGCTCAGAGCTG		

([a]) Chromosome position based on the GRCh37 assembly

([b]) *CYP2D6* PCR method included a pre-amplification of the whole gene using long template PCR using the forward (GGTAAGGGCCTGGAGCAGGAA) and reverse primer (GCCTCAACGTACCCCTGTCTC) described by Stüven et al, (12), followed by purification and subsequent sanger sequencing using the primers listed above.

Table S3. TaqMan assay ID for the common functionally validated clinically relevant SNVs.

Gene	dbSNP	TaqMan Assay ID
ABCB1	rs1045642	C___7586657_20
ABCC2	rs2273697	C__22272980_20
ABCG2	rs2231142	C__15854163_70
ABCG2	rs2231137	Custom design **(13)**
CYP2B6	rs3211371	C__30634242_40
CYP2B6	rs3745274	C___7817765_60
CYP2C19	rs12248560	C____469857_10
CYP2C19	rs4244285	C__25986767_70
CYP2C9	rs1799853	C__25625805_10
CYP2C9	rs1057910	C__27104892_10
CYP2D6	rs35742686	C__32407232_50
CYP2D6	rs3892097	C__27102431_D0
CYP2D6	rs5030656	C__32407229_60
CYP2D6	rs1065852	C__11484460_40
CYP2D6	rs28371725	C__34816116_20
CYP3A4	rs35599367	C__59013445_10
CYP3A5	rs776746	C__26201809_30
CYP4F2	rs2108622	C__16179493_40
DPYD	rs67376798	C__27530948_10
FXR	rs56163822	C__25598386_10
LXRα	rs2279238	C__15967384_10
POR	rs1057868	C___8890131_30
PXR	rs2276707	C__15882324_10
SLCO1B1	rs2306283	C___1901697_20
SLCO1B1	rs4149056	C__30633906_10
SLCO1B3	rs7311358	C__25765587_40
SLCO2B1	rs12422149	C___3101331_10
TPMT	rs1800460	C__30634116_20
TPMT	rs1142345	C_____19567_20
VDR	rs11568820	C___2880808_10
VDR	rs4516035	C___2880805_10
VKORC1	rs9923231	C__30403261_20

Statistical analysis

Analyses were performed using the software R (14) and GraphPad Prism 6 (La Jolla, USA). Differences in the proportion of possibly deleterious variants between common, low frequency, and rare or novel variant groups was assessed using a Chi-squared test (statistical significance, *P* value <0.05). Pearson correlation coefficient was used to determine association between our study allele frequencies with those reported in the ExAC database (statistical significance, two-tailed *P* value <0.05).

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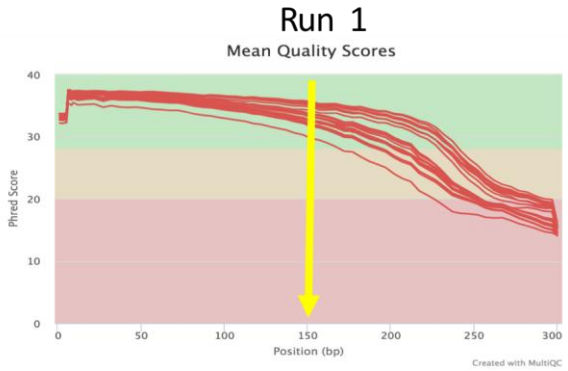
Appendix E

Figure S1. Mean Phred quality score for sequencing reads per read length. Yellow arrow indicates Phred base quality score ≥30 (99.9% sequencing accuracy) up until the 150bp read position. Base quality declines for sequencing reads that were more than 150bp in length, resulting in a mean ≥30 Phred quality score of 62.6% (Run 1), 65.1% (Run 2), and 63.2% (Run 3).



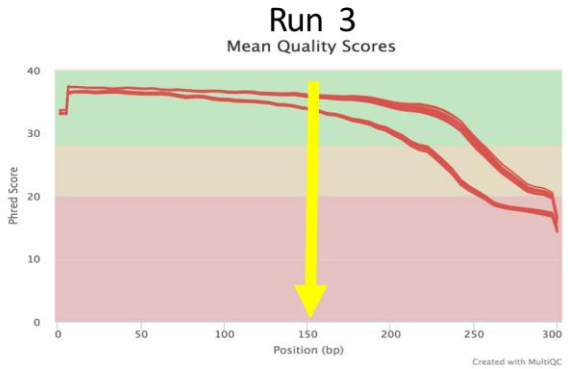


Figure S2. Frequency distribution of the percentage of guanine and cytosine nucleotides per sequenced read in 48 subjects. Arrows indicate the five samples with aberrantly higher GC content.

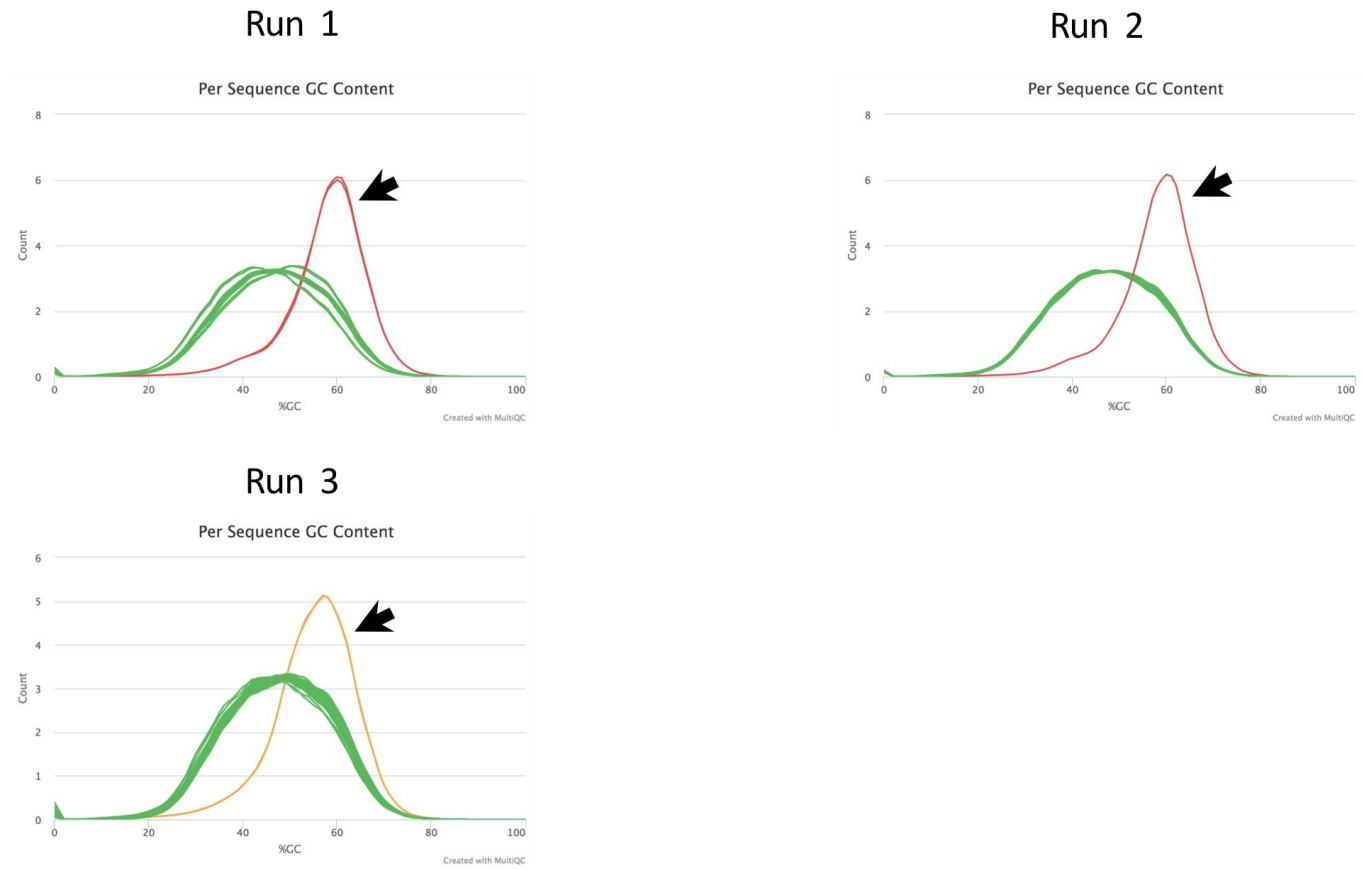


Figure S3. Mean (±SD) depth of coverage (DOC) across the targeted sequence for CES1, CBR1, CYP1A2 and showing the inaccessible target regions.

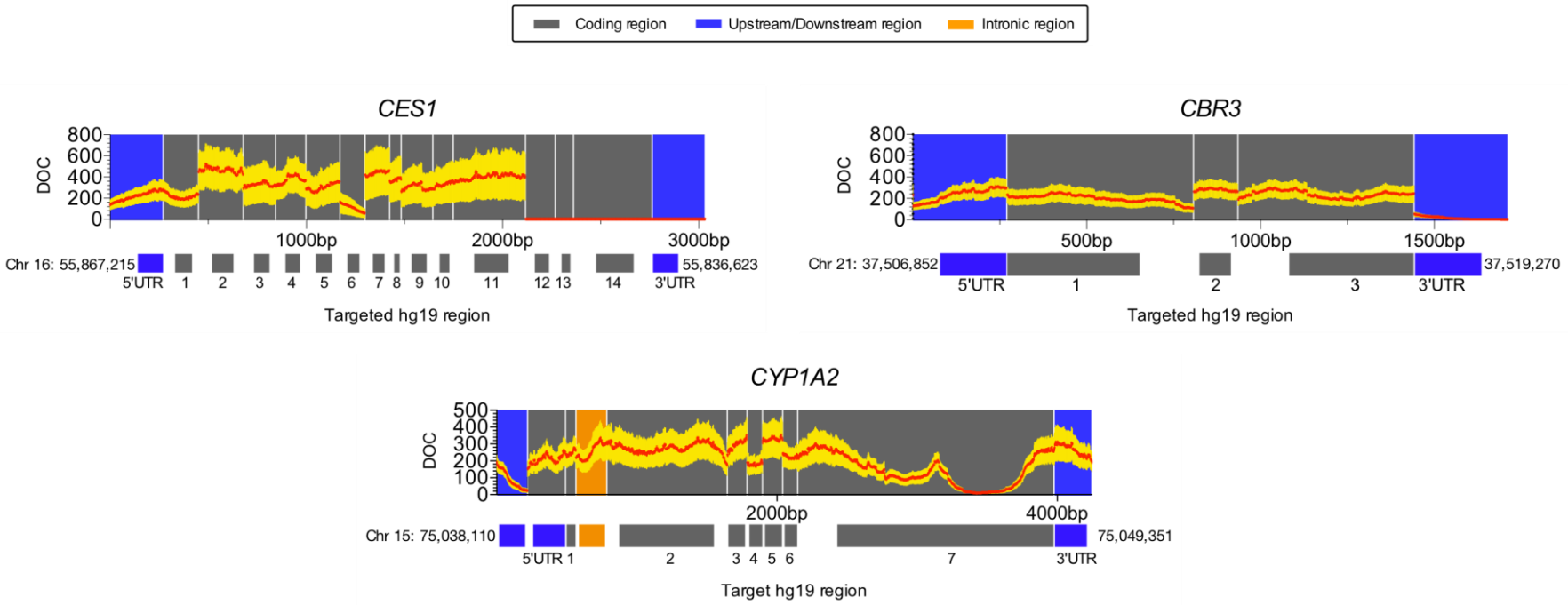


Figure S4. Data represented a mean subject DOC (±SD) across target sequence. Inter-subject and intra-subject variability of DOC across the target sequence in the validation cohort (n = 48). Matching coloured arrows indicate the five samples with aberrantly higher GC content. (‡) 1 sample repeated (PGST395).

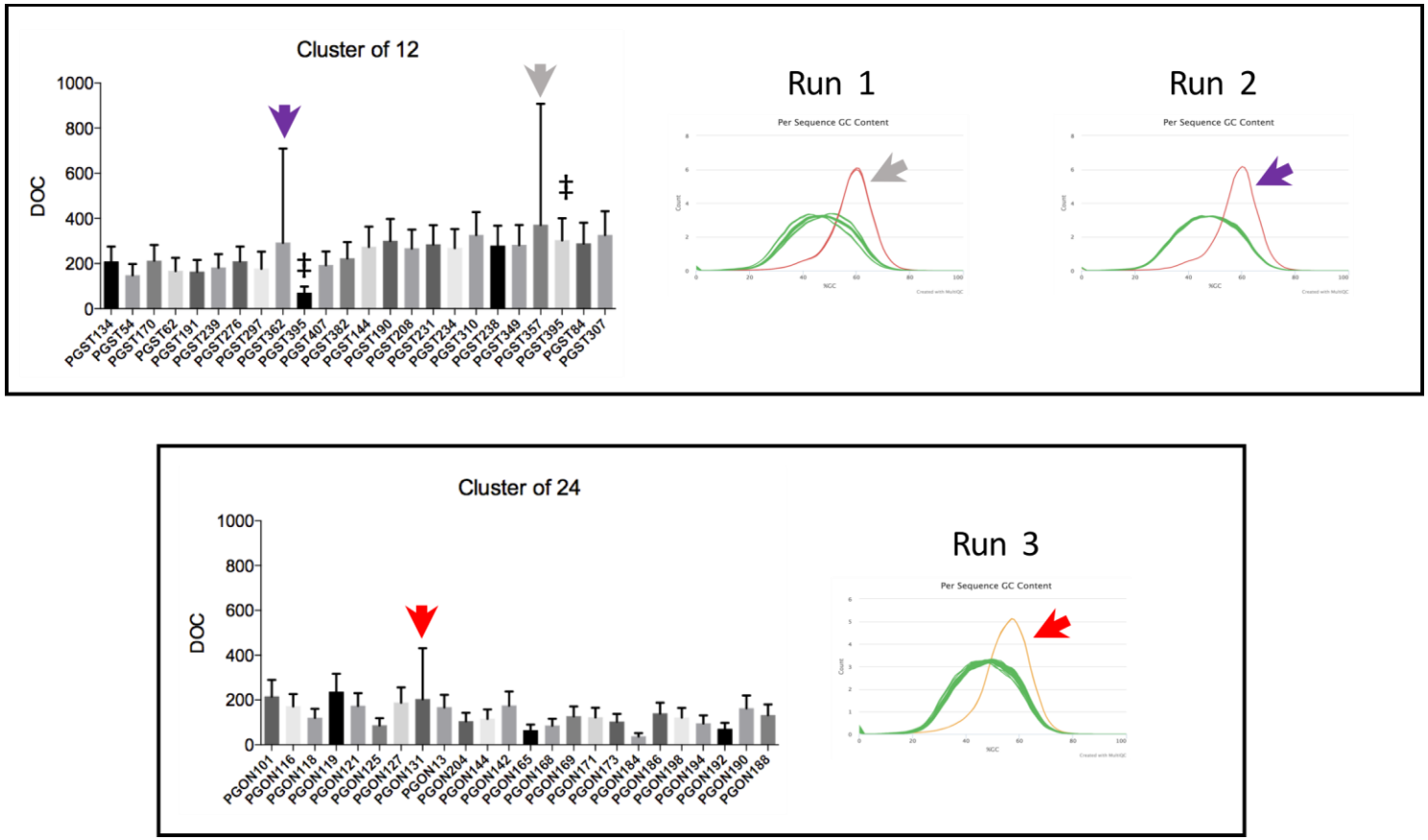


Figure S5. Similarity of observed minor allele frequencies (MAF) in relation to the reported MAF in 1000 Genomes Project (1000G) and Exome Aggregation Consortium (ExAC) datasets.

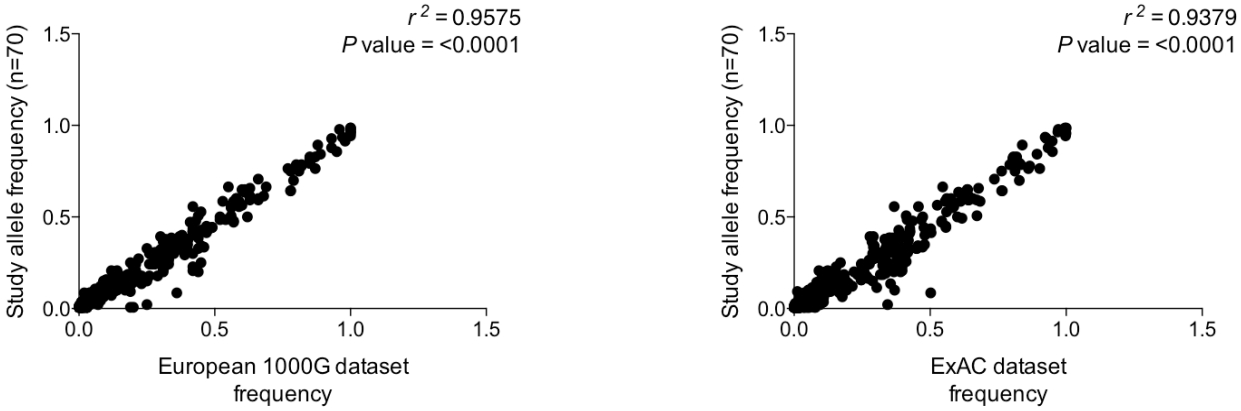
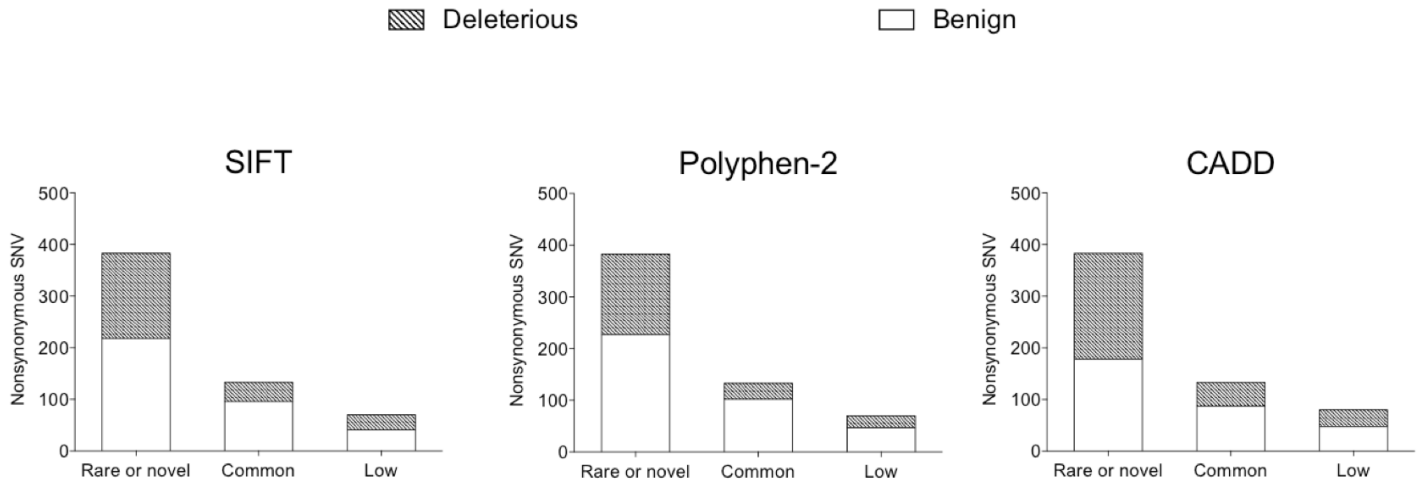


Figure S6. Rare or novel variations had greater proportion of deleterious *in silico* prediction scores (n = 245).



	SIFT			Polyphen-2			CADD		
Deleterious, n	165	37	29	156	31	23	205	46	33
Benign, n	218	96	41	227	102	47	178	87	47
Deleterious, %	43.1%	27.8%	41.4%	40.7%	23.3%	32.9%	53.5%	34.6%	41.3%
Chi-squared, *P* value		0.0076			0.0013			0.0004	

Figure S7. Zygosity of the potentially deleterious variants (CADD scaled score greater than 20) per subject (n = 245), showing there were more heterozygous variants per subject compared to homozygous. Single nucleotide variants (SNV) found in Cytochrome P450 (*CYP*) enzymes (*CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2B6*, *CYP2C19*, *CYP2C8*, *CYP2C9*, *CYP2D6*, *CYP2J2*, *CYP3A4*, *CYP3A5*, and *CYP4F2*) were highlighted in yellow.

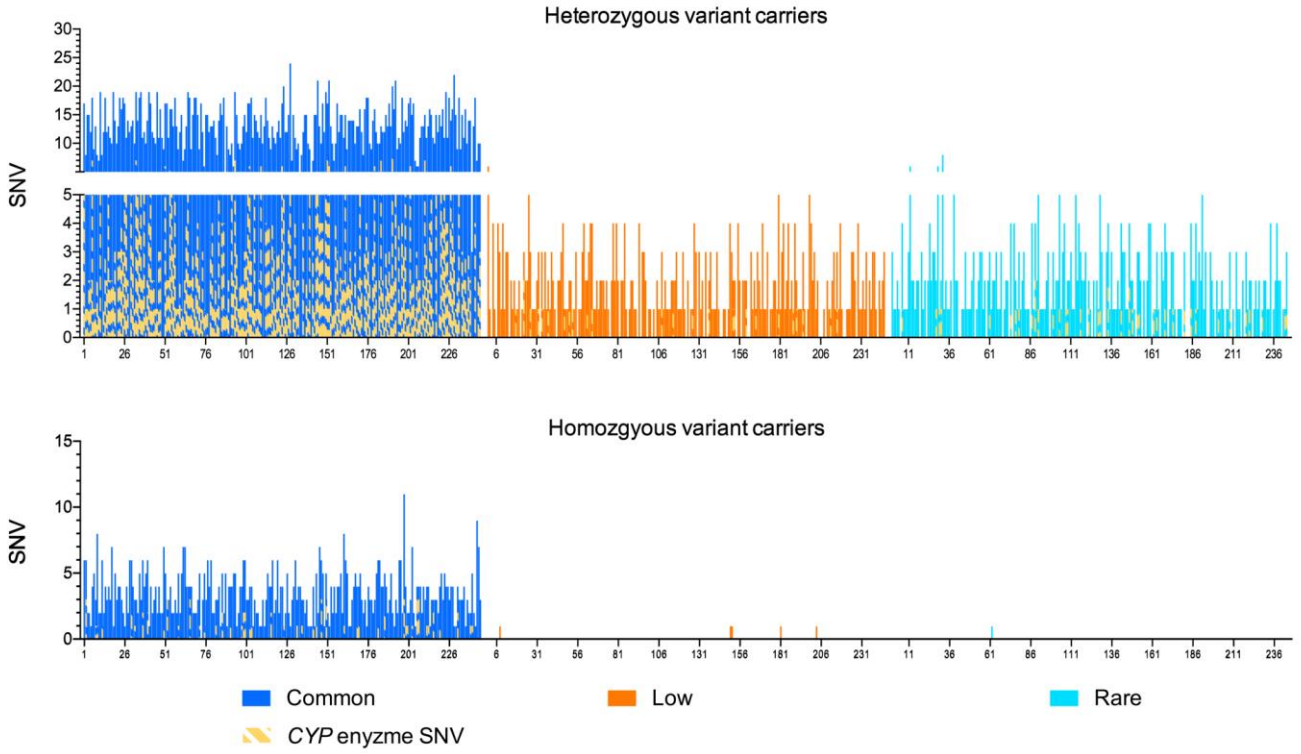


Figure S8. Number of Clinical Pharmacogenetics Implementation Consortium (CPIC) "Level 1A/1B" variants (categorized as having strong supporting evidence for affecting drug efficacy/response as well as specific prescribing recommendations https://cpicpgx.org/alleles/) found in 245 subjects separated by zygosity.

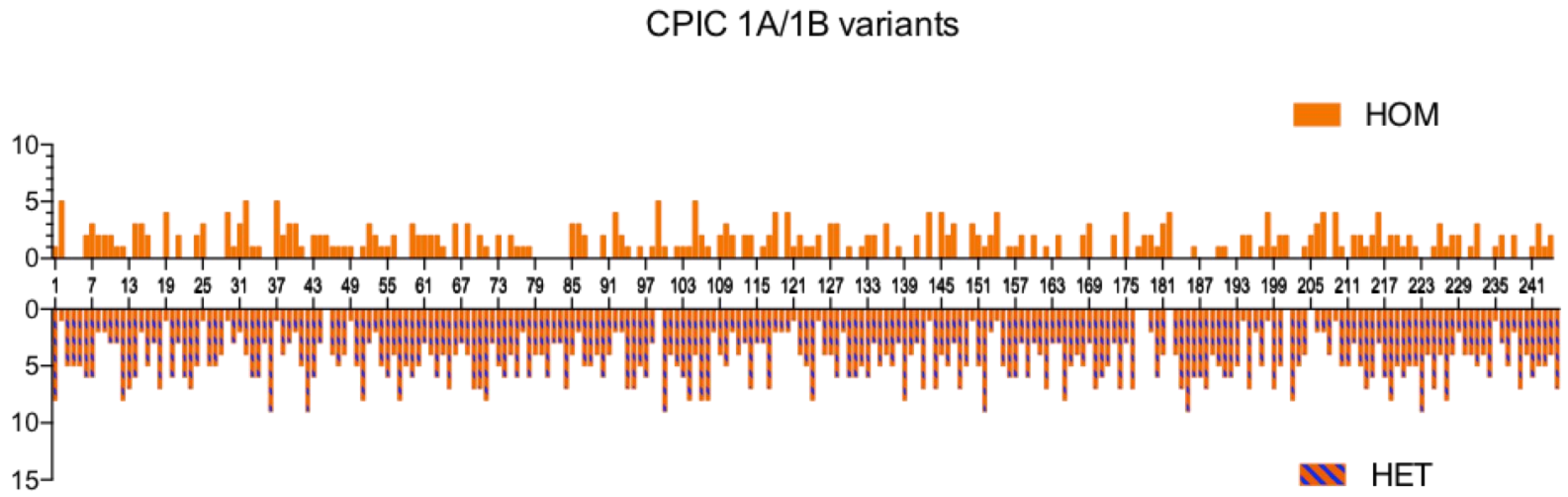


Figure S9. Histogram of the *GSTM1* and *GSTT1* gene coverage as a fraction total subject coverage in 45 subjects (excluding 3 subjects with aberrant GC content; PGST362, PGST357, PGON131).

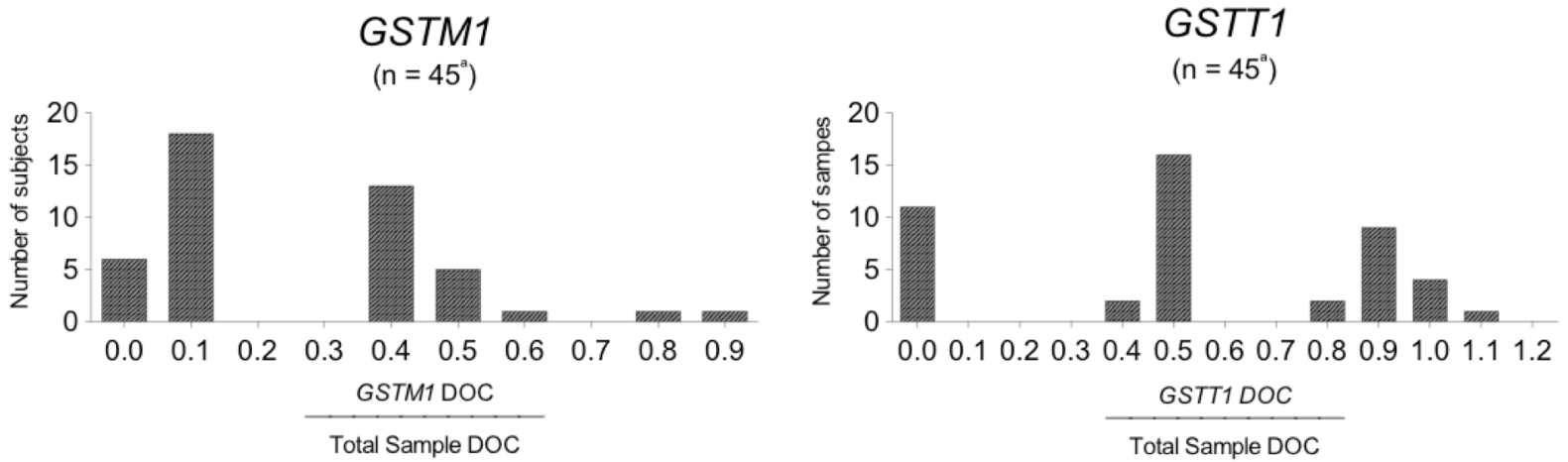
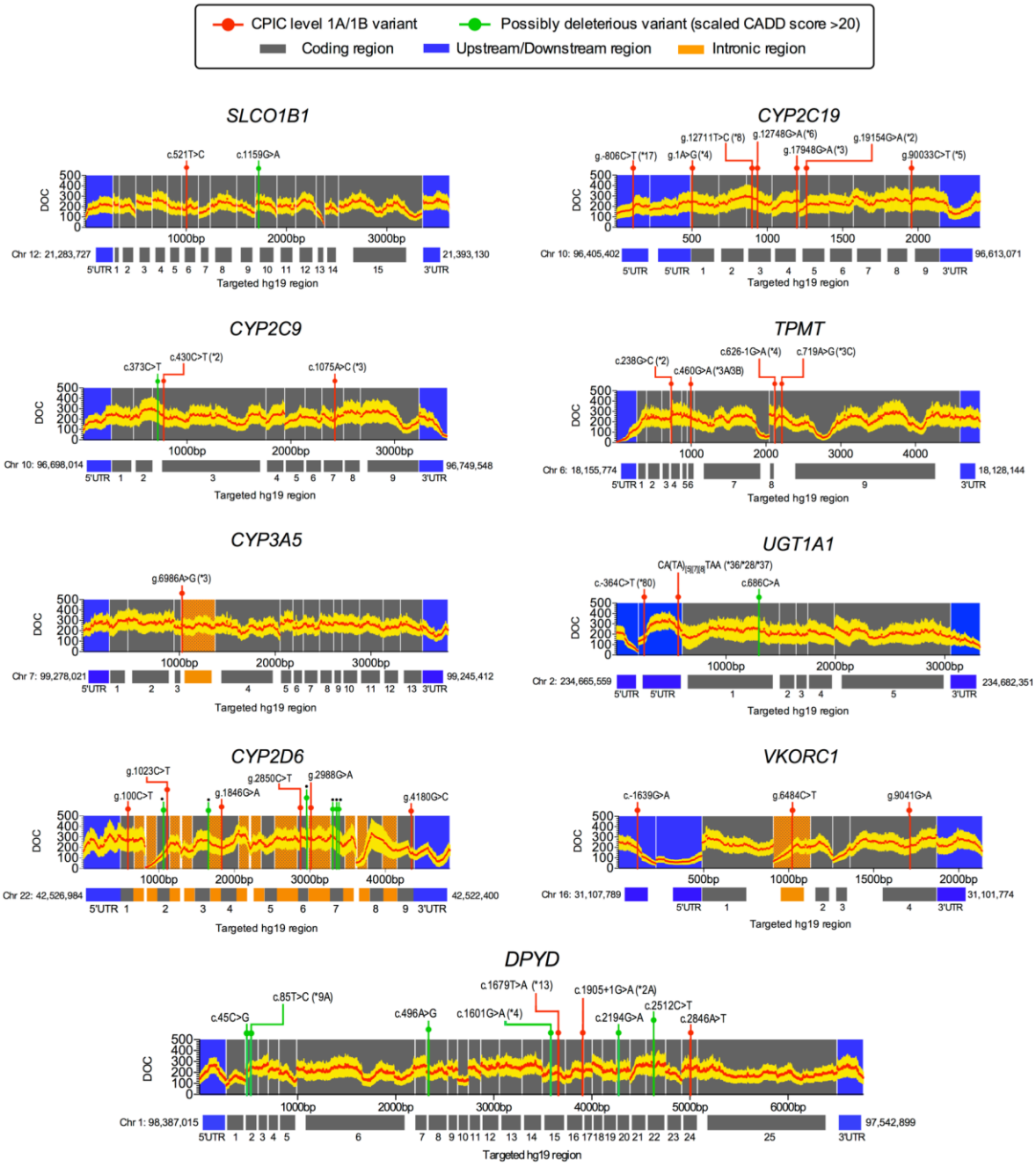


Figure S10. Mean (±SD) depth-of-coverage (DOC) across the targeted sequence of select genes (n = 24; from Sequencing Run 1 and Run 2). Vertical lines represent (red) CPIC level 1A/1B clinically actionable variant, and (green) NGS identified variants with scaled CADD score greater than 20, along with their minor allele frequencies (MAF) if found in our cohort (70 subjects). Possibly deleterious variants in CYP2D6 (●).



8 Curriculum Vitae

Name: Markus Gulilat

Post-secondary Education and Degrees:

The University of Western Ontario
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2009-2013 BMSc

The University of Western Ontario
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Honours and Awards:

Ontario Graduate Scholarship
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2013-2017

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